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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : <b>C12N 15/15, 15/58, 15/62 C12N 9/70, C07K 7/10 C12N 5/10, 1/19, 1/21 A61K 37/64, 37/54</b>		A1	(11) International Publication Number: <b>WO 91/09125</b>  (43) International Publication Date: <b>27 June 1991 (27.06.91)</b>
(21) International Application Number: <b>PCT/GB90/01911</b>  (22) International Filing Date: <b>7 December 1990 (07.12.90)</b>		(74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).	
(30) Priority data: <b>8927722.2 7 December 1989 (07.12.89) GB</b>		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.	
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(54) Title: PROTEINS AND NUCLEIC ACIDS

(57) Abstract

Relatively inactive fusion proteins are activatable by enzymes of the clotting cascade to have fibrinolytic and/or clot formation inhibition activity. For example, a fusion protein comprising two hirudin or streptokinase molecules, linked by a cleavable linkage sequence, may be cleaved to yield anti-thrombotic hirudin or fibrinolytic streptokinase by thrombin or Factor Xa. Fibrinolytic or clot formation inhibition activity is therefore directed to the site of clot formation.

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PROTEINS AND NUCLEIC ACIDS

This invention relates to proteinaceous compounds which can be cleaved to release fibrinolytic and/or anti-thrombotic activity. It also relates to nucleic acid (DNA and RNA) coding for all or part of such compounds. In preferred embodiments, the invention relates to fusion proteins produced by linking together fibrinolytic and/or anti-thrombotic proteins with a cleavable linker, their preparation, pharmaceutical compositions containing them and their use in the treatment of thrombotic disease.

The fibrinolytic system is the natural counterpart to the clotting system in the blood. In the process of blood coagulation, a cascade of enzyme activities are involved in generating a fibrin network which forms the framework of a clot, or thrombus. Degradation of the fibrin network (fibrinolysis) is accomplished by the action of the enzyme plasmin. Plasminogen is the inactive precursor of plasmin and conversion of plasminogen to plasmin is accomplished by cleavage of the peptide bond between arginine 561 and valine 562 of plasminogen. Under physiological conditions this cleavage is catalysed by tissue-type plasminogen activator (tPA) or by urokinase-type plasminogen activator (uPA).

If the balance between the clotting and fibrinolytic systems becomes locally disturbed, intravascular clots may form at inappropriate locations leading to conditions such as coronary thrombosis and myocardial infarction, deep vein thrombosis, stroke, peripheral

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1 arterial occlusion and embolism. In such cases, the  
2 administration of fibrinolytic and anti-thrombotic  
3 agents has been shown to be a beneficial therapy for  
4 the promotion of clot dissolution.

5 •

6 Fibrinolytic therapy has become relatively widespread  
7 with the availability of a number of plasminogen  
8 activators such as tPA, uPA, streptokinase and the  
9 anisoylated plasminogen streptokinase activator  
10 complex, APSAC. Each of these agents has been shown to  
11 promote clot lysis, but all have deficiencies in their  
12 activity profile which makes them less than ideal as  
13 therapeutic agents for the treatment of thrombosis  
14 (reviewed by Marder and Sherry, New England Journal of  
15 Medicine 1989, 318: 1513-1520).

16

17 A major problem shared by all of these agents is that  
18 at clinically useful doses, they are not thrombus  
19 specific as they activate plasminogen in the general  
20 circulation. The principal consequence of this is that  
21 proteins such as fibrinogen involved in blood clotting  
22 are destroyed and dangerous bleeding can occur. This  
23 also occurs with tPA despite the fact that, at  
24 physiological concentrations, it binds to fibrin and  
25 shows fibrin selective plasminogen activation.

26

27 Another important shortcoming in the performance of  
28 existing plasminogen activators is that re-occlusion of  
29 the reperfused blood vessel commonly occurs after  
30 cessation of administration of the thrombolytic agent.  
31 This is thought to be due to the persistence of  
32 thrombogenic material at the site of thrombus  
33 dissolution.

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1 Anti-thrombotic proteins may be used in the treatment  
2 or prophylaxis of thrombosis either alone or as an  
3 adjunct to fibrinolytic agents. Suitable anti-  
4 thrombotic proteins include hirudin, activated protein  
5 C and anti-thrombin III.

6 An alternative approach to enhancing fibrinolysis and  
7 inhibition of blood clotting has now been devised which  
8 is based on the use of fusion proteins cleavable to  
9 achieve release of fibrinolytic and/or anti-thrombotic  
10 activity at the site of blood clotting. To achieve  
11 this, proteins involved in fibrinolysis or inhibition  
12 of coagulation are joined by a linker region which is  
13 cleavable by an enzyme involved in blood clotting.  
14 Examples of proteins which may be incorporated into  
15 such a cleavable protein include tPA, uPA,  
16 streptokinase, plasminogen, activated protein C,  
17 hirudin and anti-thrombin III. Fusion of such proteins  
18 to a protein with a favourable property not directly  
19 related to dissolution of blood clots, for example  
20 albumin which has a long plasma half-life, may also be  
21 beneficial. An advantage of this approach is that  
22 thrombus selectivity of fibrinolytic or inhibition of  
23 clot formation activity is achieved by way of the  
24 thrombus-specific localisation of the cleaving enzymes.  
25

26 According to a first aspect of the invention, there is  
27 provided a fusion protein comprising a first sequence  
28 and a second sequence, the fusion protein being  
29 cleavable between the first and second sequences by an  
30 enzyme involved in blood clotting, wherein after the  
31 fusion protein is so cleaved the first and second  
32 sequences, or either of them, has greater fibrinolytic  
33 sequences,

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1 and/or anti-thrombotic activity than the uncleaved  
2 fusion protein.

3

4 The fusion protein may be a cleavable dimer of two  
5 fibrinolytic and/or anti-thrombotic proteins, such as  
6 hirudin or streptokinase. It may be a homodimer or a  
7 heterodimer. The fusion protein may have substantially  
8 reduced or no fibrinolytic and/or anti-thrombotic  
9 activity compared to the cleavage products, but a  
10 certain amount of activity in the fusion protein can be  
11 tolerated. It is not necessary for both the cleavage  
12 products to have fibrinolytic and/or anti-thrombotic  
13 activity, but it is preferred for them to do so.  
14

15 The fusion protein is not restricted to being a dimer;  
16 it may have any number (such as three, four or more)  
17 sequences which are cleavable one from the other,  
18 compatible with the therapeutic utility of the protein.  
19 At least one, and preferably more than one or even all,  
20 of the sequences resulting from the cleavage will have  
21 greater activity than the fusion protein, or a  
22 combination of some or all of the cleavage products  
23 will collectively have such greater activity. In any  
24 event, cleavage will result in a net increase in or  
25 release of activity.  
26

27 Proteinaceous compounds in accordance with the first  
28 aspect of the invention, are therefore cleaved to  
29 release activity in at least one of two ways. First, a  
30 compound may be cleaved to release fibrinolytic  
31 activity. Secondly, a compound may be cleaved to  
32 release anti-thrombotic activity. Conceivably, a  
33 compound may be cleaved to release both functions. It

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1 should be noted that a released fragment of the fusion  
2 protein may have fibrinolytic activity directly (in  
3 that it lyses fibrin) or indirectly (in that it causes  
4 activation of a molecule which leads to lysis of  
5 fibrin).

6 One preferred proteinaceous compound which is cleavable  
7 to have enhanced anti-thrombotic activity is a fusion  
8 protein of two hirudin molecules linked (for example  
9 carboxy terminus to amino terminus) by a linker amino  
10 acid sequence cleavable, for example, by Factor Xa.  
11

12 Hirudins are naturally occurring polypeptides of 65 or  
13 66 amino acids in length that are produced by the leech  
14 Hirudo medicinalis. Hirudin is an anticoagulating  
15 agent which binds to thrombin and prevents blood  
16 coagulation by inhibiting thrombin from catalysing the  
17 conversion of fibrinogen to fibrin, thus preventing the  
18 formation of the protein framework of blood clots. The  
19 binding of hirudin also prevents other prothrombic  
20 activities of thrombin including activation of factors  
21 V, VII, XIII and platelets. There are three principal  
22 variants of hirudin (named HV-1, HV-2 and HV-3).  
23

24 Another preferred fusion protein comprises two  
25 streptokinase molecules linked (for example carboxy  
26 terminus to amino terminus) by a linker amino acid  
27 sequence cleavable, for example, by thrombin.  
28

29 Streptokinase is a 414 amino acid, 47kDa protein  
30 secreted by many pathogenic streptococci of different  
31 serogroups. It is a plasminogen activator but, unlike  
32 mammalian plasminogen activators, it is not a protease  
33

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1 and it activates plasminogen by forming a binary  
2 complex with plasminogen (SK-plasminogen) which  
3 functions as an activator of free plasminogen.  
4 Streptokinase is effective in inducing clot lysis in  
5 the treatment of myocardial infarction and is widely  
6 used for this indication.

7

8 Cleavable fusion proteins within the scope of this  
9 invention may have reduced fibrinolytic and/or  
10 anti-thrombotic activity compared to their component  
11 molecules; cleavage releases the component molecules  
12 which possess to an adequate degree the activity of  
13 their wild-type parent molecules.

14

15 The blood coagulation mechanism comprises a series of  
16 enzyme reactions which culminate in the production of  
17 insoluble fibrin, which forms the mesh-like protein  
18 framework of blood clots. Thrombin is the enzyme  
19 responsible for the conversion of soluble fibrinogen to  
20 fibrin. Conversion of prothrombin, the inactive  
21 precursor of thrombin, to thrombin is catalysed by  
22 activated Factor X (Factor Xa). (Thrombin is also  
23 known as Factor IIa, and prothrombin as Factor II.)

24

25 Factor Xa is generated from Factor X extrinsically or  
26 intrinsically. In the extrinsic route, Factor VII is  
27 activated to Factor VIIa, which generates Factor Xa  
28 from Factor X. In the intrinsic route, the activation  
29 of Factor X to Factor Xa is catalysed by Factor IXa.  
30 Factor IXa is generated from Factor IX by the action of  
31 Factor XIa, which in turn is generated by the action of  
32 Factor XIIa on Factor XI. Factor XIIa is generated  
33 from Factor XII by the action of Kallikrein. Factors

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1       VIIIa and Va are thought to act as cofactors in the  
2       activation of Factors X and II, respectively.

3  
4       Fibrin, as first formed from fibrinogen, is in the  
5       loose form. Loose fibrin is converted to tight fibrin  
6       by the action of Factor XIIIa, which crosslinks fibrin  
7       molecules.

8  
9       Activated protein C is an anticoagulant serine protease  
10      generated in the area of clot formation by the action  
11      of thrombin, in combination with thrombomodulin, on  
12      protein C. Activated protein C regulates clot  
13      formation by cleaving and inactivating the  
14      pro-coagulant cofactors Va and VIIIa.

15  
16      The term "enzyme involved in blood clotting" as used in  
17      this specification therefore includes kallikrein  
18      Factors XIIa, XIa, IXa, VIIa, Xa and thrombin (Factor  
19      IIa), which are directly involved in the formation of  
20      fibrin and activated protein C, which is involved in  
21      the control of blood clotting. The most preferred  
22      enzymes are Factor Xa and thrombin because they are  
23      most immediately involved with fibrin formation.

24  
25      Generation and activity of at least Factor Xa and  
26      thrombin is tightly regulated to ensure that thrombus  
27      generation is restricted to the site of the  
28      thrombogenic stimulus. This localisation is achieved by  
29      the combined operation of at least two control  
30      mechanisms: the blood clotting enzymes function as  
31      complexes intimately associated with the phospholipid  
32      cellular membranes of platelets and endothelial cells  
33      at the site of vascular injury (Mann, K. G., 1984, in:

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1 "Progress in Hemostasis and Thrombosis", 1 - 24, ed  
2 Spaet, T. H. Grune and Stratton); and, free thrombin or  
3 Factor Xa released from the thrombus site into the  
4 circulation is rapidly inactivated by the action of  
5 proteinase inhibitors such as anti-thrombin III.  
6

7 Thus, the activity of the penultimate (Factor Xa) and  
8 the final (thrombin) enzymes in the clotting cascade  
9 are particularly well localised to the site of thrombus  
10 generation and for this reason are preferred.

11 Thrombin has been found to remain associated with  
12 thrombi and to bind non-covalently to fibrin. On  
13 digestion of thrombi with plasmin, active thrombin is  
14 liberated and is thought to contribute to the  
15 reformation of thrombi and the re-occlusion of vessels  
16 which commonly occurs following thrombolytic treatment  
17 with plasminogen activators (Bloom A. L., 1962, Br. J.  
18 Haematol., 82, 129; Francis et al, 1983, J. Lab. Clin. Med., 102, 220; Mirshahi et al, 1989, Blood 74, 1025).  
19

20  
21 For these reasons, it is preferred in certain  
22 embodiments of the invention to produce fusion proteins  
23 activatable by thrombin or Factor Xa thereby to create  
24 a preferred class of thrombus-selective, fibrinolytic  
25 proteins. The most preferred of these fusion proteins  
26 regain the favourable properties of the parent  
27 molecules upon cleavage and exhibit thrombus  
28 selectivity by the novel property of being cleaved to  
29 release the component proteins of the fusion protein at  
30 the site of new thrombus formation by the action of one  
31 of the enzymes involved in generation of the thrombus  
32 and preferably localised there.  
33

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1 Factor Xa (E.C.3.4.21.6) is a serine protease which  
2 converts human prothrombin to thrombin by specific  
3 cleavage of the Arg(273)-Thr(274) and Arg(322)-Ile(323)  
4 peptide bonds (Mann et al 1981, Methods in Enzymology  
5 80 286-302). In human prothrombin, the Arg(273)-  
6 Thr(274) site is preceded by the tripeptide Ile-Glu-Gly  
7 and the Arg(322)-Ile(323) site is preceded by the  
8 tripeptide Ile-Asp-Gly. The structure required for  
9 recognition by Factor Xa appears to be determined by  
10 the local amino acid sequence preceding the cleavage  
11 site (Magnusson et al, 1975, in: "Proteases and  
12 Biological Control", 123-149, eds., Reich et al, Cold  
13 Spring Harbor Laboratory, New York). Specificity for  
14 the Ile-Glu-Gly-Arg and Ile-Asp-Gly-Arg sequence is not  
15 absolute as Factor Xa has been found to cleave other  
16 proteins, for example Factor VIII at positions 336,  
17 372, 1689 and 1721, where the preceding amino acid  
18 sequence differs significantly from this format (Eaton  
19 et al, 1986 Biochemistry 25 505-512). As the principal  
20 natural substrate for Factor Xa is prothrombin,  
21 preferred recognition sequences are those in which  
22 arginine and glycine occupy the P1 and P2 positions,  
23 respectively, an acidic residue (aspartic or glutamic  
24 acid) occupies the P3 position and isoleucine or  
25 another small hydrophobic residue (such as alanine,  
26 valine, leucine or methionine) occupies the P4  
27 position. However, as Factor Xa can cleave sequences  
28 which differ from this format, other sequences  
29 cleavable by Factor Xa may be used in the invention, as  
30 can other sequences cleavable by other enzymes of the  
31 clotting cascade.

32

33

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1    In order to make fusion proteins which are cleavable by  
2    these preferred enzymes, the amino acid sequence  
3    linking the components of the fusion protein must be  
4    recognised as a cleavage site for these preferred  
5    enzymes. To make fusion proteins which are cleaved  
6    by, for example, Factor Xa, an amino acid sequence  
7    cleavable by Factor Xa may be used to link the two  
8    components (that is, the first and second, and possibly  
9    other, sequences) of the fusion protein. The sequence  
10   Ile-Glu-Gly-Arg which is at one of the sites in  
11   prothrombin cleaved by Factor Xa may be such a  
12   sequence. Other possibilities would be sequences or  
13   mimics of sequences cleaved by Factor Xa in other  
14   proteins or peptides. DNA coding for the  
15   Ile-Glu-Gly-Arg sequence as the carboxy-terminal part  
16   of a cleavable linker as a protein production aid is  
17   disclosed in UK Patent Application GB-A-2160206 but the  
18   use of an Ile-Glu-Gly-Arg sequence for the purpose of  
19   this invention is not disclosed in that specification.  
20

21   Cleavage of fusion proteins by an enzyme of the  
22   clotting cascade such as thrombin or Factor Xa can be  
23   measured in a number of ways, for example by SDS-PAGE  
24   analysis, and by assaying for the functions of one or  
25   more of the cleavage products of the fusion protein.  
26

27   Thrombin (E.C. 3.4.21.5) is a serine protease which  
28   catalyses the proteolysis of a number of proteins  
29   including fibrinogen (A alpha and B beta chains),  
30   Factor XIII, Factor V, Factor VII, Factor VIII, protein  
31   C and anti-thrombin III. The structure required for  
32   recognition by thrombin appears to be partially  
33   determined by the local amino acid sequence around the

1 cleavage site but is also determined to a variable  
2 extent by sequence(s) remote from the cleavage site.  
3 For example, in the fibrinogen A alpha chain, residues  
4 P2 (Val), P9 (Phe) and P10 (Asp) are crucial for  
5  $\alpha$ -thrombin-catalysed cleavage at the Arg(16)-Gly(17)  
6 peptide bond (Ni, F. *et al* 1989, Biochemistry 28  
7 3082-3094). Comparative studies of several proteins  
8 and peptides which are cleaved by thrombin has led to  
9 the proposal that optimum cleavage sites for  $\alpha$ -thrombin  
10 may have the structure of (i) P4-P3-Pro-Arg-P1'-P2',  
11 where each of P3 and P4 is independently a hydrophobic  
12 amino acid (such as valine) and each of P1' and P2' is  
13 independently a non-acidic amino acids, or (ii)  
14 P2-Arg-P1' where P2 or P1' is glycine (Chang, J. 1985,  
15 Eur. J. Biochem. 151 217-224). There are, however,  
16 exceptions to these general structures which are  
17 cleaved by thrombin and which may be used in the  
18 invention.

19 To produce a fusion protein which could be cleaved by  
20 thrombin, a linker sequence containing a site  
21 recognised and cleaved by thrombin may be used. An  
22 amino acid sequence such as that cleaved by thrombin in  
23 the fibrinogen A alpha chain may be used. Other  
24 possible sequences would include those involved in the  
25 cleavage by thrombin of fibrinogen B beta, Factor XIII,  
26 Factor V, Factor VII, Factor VIII, protein C,  
27 anti-thrombin III and other proteins whose cleavage is  
28 catalysed by thrombin. An example of a thrombin  
29 cleavable linker may be the sequence Gly-Pro-Arg which  
30 is identical to that found at positions 17-20 in  
31 fibrinogen A alpha. This is not the principal thrombin  
32 cleavage site in fibrinogen A alpha but thrombin can

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1 cleave the Arg(19)-Val(20) peptide bond. Another  
2 suitable thrombin cleavable linker sequence is  
3 Val-Glu-Leu-Gln-Gly-Val-Val-Pro-Arg which is identical  
4 to that found in Factor XIII.

5

6 In a preferred embodiment the invention relates to  
7 fusion proteins of streptokinase and/or hirudin linked  
8 by peptide sequences which are cleaved by thrombin,  
9 Factor Xa or other enzymes involved in blood clotting  
10 to release products with fibrinolytic and/or anti-  
11 thrombotic activity.

12

13 Fusion proteins in accordance with the invention may  
14 contain other modifications (as compared to wild-type  
15 counterparts of their components such as streptokinase  
16 and hirudin) which may be one or more additions,  
17 deletions or substitutions. An example of such a  
18 modification would be streptokinase variants in which  
19 inappropriate glycosylation during yeast expression was  
20 prevented by substitution of sequences recognised as  
21 glycosylation signals by yeast. Another example would  
22 be the addition of an Arg-Gly-Asp-Xaa sequence, where  
23 Xaa represents a variable amino acid such as Ser, to  
24 the carboxy terminus of the fusion to enhance its  
25 plasma lifetime.

26

27 Preferred features of fusion proteins within the scope  
28 of the invention also apply, where appropriate, to  
29 other compounds of the invention, mutatis mutandis.

30

31 Fusion proteins in accordance with the first aspect of  
32 the invention can be synthesised by any convenient  
33 route. According to a second aspect of the invention

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1 there is provided a process for the preparation of a  
2 proteinaceous compound as described above, the process  
3 comprising coupling successive amino acid residues  
4 together and/or ligating oligopeptides. Although  
5 proteins may in principle be synthesised wholly or  
6 partly by chemical means, the route of choice will be  
7 ribosomal translation, preferably in vivo, of a  
8 corresponding nucleic acid sequence. The protein may  
9 be glycosylated appropriately.

10 It is preferred to produce proteins in accordance with  
11 the invention by using recombinant DNA technology. DNA  
12 encoding each of the first and second sequences of the  
13 fusion protein may be from a cDNA or genomic clone or  
14 may be synthesised. Amino acid substitutions,  
15 additions or deletions are preferably introduced by  
16 site-specific mutagenesis. Suitable DNA sequences  
17 encoding streptokinase and hirudin and other  
18 polypeptide sequences useful in the scope of the  
19 invention may be obtained by procedures familiar to  
20 those having ordinary skill in genetic engineering.  
21 For several proteins, it is a routine procedure to  
22 obtain recombinant protein by inserting the coding  
23 sequence into an expression vector and transfecting or  
24 transforming the vector into a suitable host cell. A  
25 suitable host may be a bacterium such as E. coli, a  
26 eukaryotic microorganism such as yeast or a higher  
27 eukaryotic cell.

28  
29 According to a third aspect of the invention, there is  
30 provided synthetic or recombinant nucleic acid coding  
31 for a proteinaceous compound as described above. The  
32 nucleic acid may be RNA or DNA. Preferred

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1 characteristics of this aspect of the invention are as  
2 for the first aspect.

3

4 According to a fourth aspect of the invention, there is  
5 provided a process for the preparation of nucleic acid  
6 in accordance with the third aspect, the process  
7 comprising coupling successive nucleotides together  
8 and/or ligating oligo- and/or polynucleotides.

9

10 Recombinant nucleic acid in accordance with the third  
11 aspect of the invention may be in the form of a vector,  
12 which may for example be a plasmid, cosmid or phage.  
13 The vector may be adapted to transfect or transform  
14 prokaryotic (for example bacterial) cells and/or  
15 eukaryotic (for example yeast or mammalian) cells. A  
16 vector will comprise a cloning site and usually at  
17 least one marker gene. An expression vector will have  
18 a promoter operatively linked to the sequence to be  
19 inserted into the cloning site and, preferably, a  
20 sequence enabling the protein product to be secreted.  
21 Expression vectors and cloning vectors (which need not  
22 be capable of expression) are included in the scope of  
23 the invention.

24

25 It is to be understood that the term "vector" is used  
26 in this specification in a functional sense and is not  
27 to be construed as necessarily being limited to a  
28 single nucleic acid molecule.

29

30 Using a vector, for example as described above, fusion  
31 proteins in accordance with the invention may be  
32 expressed and secreted into the cell culture medium in  
33 a biologically active form without the need for any

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1 additional biological or chemical procedures. Suitable  
2 cells or cell lines to be transformed may be mammalian  
3 cells which grow in continuous culture and which can be  
4 transfected or otherwise transformed by standard  
5 techniques. Examples of suitable cells include Chinese  
6 hamster ovary (CHO) cells, mouse myeloma cell lines  
7 such as P3X63-Ag8.653, COS cells, HeLa cells, BHK  
8 cells, melanoma cell lines such as the Bowes cell line,  
9 mouse L cells, human hepatoma cell lines such as Hep  
10 G2, mouse fibroblasts and mouse NIH 3T3 cells. Such  
11 cells may be particularly appropriate for expression  
12 when one or more of the protein sequences constituting  
13 the fusion protein is of mammalian derivation, such as  
14 tissue plasminogen activator (t-PA).

15  
16 Yeast (for example Pichia pastoris or Saccharomyces  
17 cerevisiae) or bacteria (for example Escherichia coli)  
18 may be preferred for the expression of many of the  
19 fusion proteins of the invention, as may insect cells  
20 such as those which are Baculovirus-infected.

21  
22 Compounds of the present invention may be used within  
23 pharmaceutical compositions for the prevention or  
24 treatment of thrombosis or other conditions where it is  
25 desired to produce local fibrinolytic and/or  
26 anticoagulant activity. Such conditions include  
27 myocardial and cerebral infarction, arterial and venous  
28 thrombosis, thromboembolism, post-surgical adhesions,  
29 thrombophlebitis and diabetic vasculopathies.

30  
31 According to a fifth aspect of the invention, there is  
32 provided a pharmaceutical composition comprising one or  
33 more compounds in accordance with the first aspect of

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1 the invention and a pharmaceutically or veterinarily  
2 acceptable carrier. Such a composition may be adapted  
3 for intravenous administration and may thus be sterile.  
4 Examples of compositions in accordance with the  
5 invention include preparations of sterile fusion  
6 proteins in isotonic physiological saline and/or  
7 buffer. The composition may include a local  
8 anaesthetic to alleviate the pain of injection.  
9 Compounds of the invention may be supplied in unit  
10 dosage form, for example as a dry powder or water-free  
11 concentrate in a hermetically sealed container such as  
12 an ampoule or sachet indicating the quantity of  
13 protein. Where a compound is to be administered by  
14 infusion, it may be dispensed by means of an infusion  
15 bottle containing sterile water for injections or  
16 saline or a suitable buffer. Where it is to be  
17 administered by injections, it may be dispensed with an  
18 ampoule of water for injection, saline or a suitable  
19 buffer. The infusible or injectable composition may be  
20 made up by mixing the ingredients prior to  
21 administration. Where it is to be administered as a  
22 topical treatment, it may be dispensed in a suitable  
23 base.

24

25 The quantity of material to be administered will depend  
26 on the amount of fibrinolysis or inhibition of clotting  
27 required, the required speed of action, the seriousness  
28 of the thromboembolic position and the size of the  
29 clot. The precise dose to be administered will, because  
30 of the very nature of the condition which compounds of  
31 the invention are intended to treat, be determined by  
32 the physician. As a guideline, however, a patient  
33 being treated for a mature thrombus will generally

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1 receive a daily dose of a fusion protein of from 0.01  
2 to 10 mg/kg of body weight either by injection in for  
3 example up to 5 doses or by infusion.

4  
5 The invention may be used in a method for the treatment  
6 or prophylaxis of thrombosis, comprising the  
7 administration of an effective non-toxic amount of a  
8 compound in accordance with the first aspect.  
9 According to a further aspect of the invention, there  
10 is therefore provided the use of a compound as  
11 described above in the preparation of a thrombolytic  
12 and/or anticoagulant agent.

13  
14 The invention concerns especially the DNAs, the  
15 vectors, the transformed host strains, the fusion  
16 proteins and the process for the preparation thereof as  
17 described in the examples.

18  
19 The following examples of the invention are offered by  
20 way of illustration, and not by way of limitation. The  
21 examples refer to the accompanying drawings, in which:

22  
23 Figure 1 shows schematically the arrangement of a  
24 set of oligonucleotides used in the assembly of a  
25 synthetic hirudin gene (Preparation 1);

26  
27 Figure 2 shows a map of plasmid pSW6 (Preparation  
28 2);

29

30  
31 Figure 3 shows a map of plasmid pJK1 (Preparation  
32 2);

33

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1       Figure 4 shows a map of plasmid pGC517 (Example  
2       4);  
3

4       Figure 5 shows a zymograph of E. coli strains  
5       expressing streptokinase activity (Example 11);  
6       and  
7

8       Figure 6 shows a zymograph demonstrating cleavage  
9       of a streptokinase-streptokinase fusion protein by  
10      thrombin (Example 13).  
11

12      Methodology  
13

14      The techniques of genetic engineering and genetic  
15      manipulation used in the manufacture of the genes  
16      described and in their further manipulation for  
17      construction of expression vectors are well known to  
18      those skilled in the art. Descriptions of modern  
19      techniques can be found in the laboratory manuals  
20      "Current Protocols in Molecular Biology", Volumes 7  
21      and 2, edited by F. M. Ausubel *et al.*, published by  
22      Wiley-Interscience, New York and in "Molecular Cloning,  
23      A Laboratory Manual" (second edition) edited by  
24      Sambrook, Fritsch and Maniatis published by Cold  
25      Spring Harbor Laboratories, New York. M13mp18, M13mp19  
26      and pUC19 DNAs were purchased from Pharmacia Ltd.,  
27      Midsummer Boulevard, Central Milton Keynes, Bucks, MK9  
28      3HP, United Kingdom. Restriction endonucleases were  
29      purchased either from Northumbria Biologicals Limited,  
30      South Nelson Industrial Estate, Cramlington,  
31      Northumberland, NE23 9HL, United Kingdom or from New  
32      England Biolabs, 32 Tozer Road, Beverly, MA 01915-5510  
33      USA. E. coli HW1110 (lacIq) is used as expression host

1 in certain of the following examples: a suitable  
2 commercially available alternative is JM109, available  
3 from Northumbria Biologicals Ltd.

4

5 PREPARATION 1 - Construction of a Hirudin HV1 gene

6

7 A. Gene Design

8

9 A synthetic hirudin HV-1 gene was designed based on the  
10 published amino acid sequence (Dodd J., et al FEBS  
11 Letters 165 180 (1984)). Unique restriction  
12 endonuclease target sites were incorporated to  
13 facilitate subsequent genetic manipulation (see SEQ. ID  
14 NO:1 in the Sequence Listings immediately before the  
15 claims). The codons selected were those favoured by  
16 either S. cerevisiae or E. coli and are thus suitable  
17 for expression in either organism.

18

19 B. Gene Construction

20

21 The gene sequence was divided into 12 oligodeoxyribo-  
22 nucleotides (see SEQ. ID NO:2) such that after  
23 annealing each complementary pair 2 oligonucleotides,  
24 they were left with cohesive ends either for or of 7  
25 bases in length.

26

27 C. Oligonucleotide Synthesis

28

29 The oligonucleotides were synthesised by automated  
30 phosphoramidite chemistry on an Applied Bio-Systems  
31 380B DNA Synthesiser, using cyanoethyl  
32 phosphoramidites. The methodology is now widely used  
33 and has already been described (Beaucage, S.L. and

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1      Caruthers, M.H. Tetrahedron Letters 24, 245 (1981) and  
2      Caruthers, M. H. Science 230, 281-285 (1985)).  
3

4      D. Gene Assembly  
5

6      The oligonucleotides were kinased to provide them with  
7      a 5' phosphate to allow their subsequent ligation. The  
8      oligonucleotides were assembled as shown in Figure 1.  
9

10     Kinasing of Oligomers  
11

12     100 pmole of oligomer was dried down and resuspended in  
13     20 µl kinase buffer (70 mM Tris, pH 7.6, 10 mM MgCl<sub>2</sub>,  
14     1 mM ATP, 0.2 mM spermidine, 0.5 mM dithiothreitol  
15     (DTT)). T4 polynucleotide kinase (2 µl. 10 000 U/ml)  
16     was added and the mixture was incubated at 37°C for 30  
17     minutes. The kinase was then inactivated by heating at  
18     70°C for 10 minutes.  
19

20     Complementary pairs of kinased oligonucleotides were  
21     annealed in pairs (90°C, 5 minutes, followed by slow  
22     cooling at room temperature). The 6 paired oligomers  
23     were then mixed together, incubated at 50°C for  
24     5 minutes and allowed to cool. They were then ligated  
25     overnight at 16°C with T4 DNA ligase. The strategy is  
26     shown diagrammatically in Figure 1 (note  
27     P = 5'-phosphate). To prevent possible multi-  
28     merisation, oligomers designated BB2011 and BB2020  
29     were not kinased. The sequences of the oligomers shown  
30     in Figure 1 correspond to those given in SEQ.ID NO:2.  
31

32     The ligation products were separated on a 2% low  
33     gelling temperature agarose gel and the DNA fragment of

1    ca. 223 base pairs corresponding to the hirudin HV-1  
2    gene was excised and extracted from the gel. The  
3    purified fragment was then ligated to HindIII and EcoRI  
4    treated pUC19 plasmid DNA. The transformation of E.  
5    coli host strains was accomplished using standard  
6    procedures. The strain used as a recipient in the  
7    transformation of plasmid vectors was HW87 which has  
8    the following genotype:

9  
10      araD139(ara-leu)DELT A7697 (lacIPOZY)DELT A74 galU  
11  
12      galK hsdR rpsL srl recA56

13  
14    The use of HW87 was not critical: any suitable  
15    recipient strain could be used, for example, E. coli  
16    AG1, which is available from Northumbria Biologicals  
17    Ltd. The recombinant ligation products were  
18    transformed into E. coli K12 host strain HW87 and  
19    plated onto Luria-agar ampicillin (100 µg/ml) plates.  
20    Twelve ampicillin-resistant colonies were picked and  
21    used to prepare plasmid DNA for sequence analysis.  
22    Double stranded dideoxy sequence analysis using a  
23    universal sequencing primer BB22  
24    (5'-CAGGGTTTCCAGTCACG-3'), (SEQ ID NO:3)  
25    complementary to the universal primer region of pUC19  
26    was used to identify a correct clone pUC19 HV-1.  
27    The pUC19 recombinant was used to construct an  
28    expression vector.

29  
30  
31  
32  
33

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1    PREPARATION 2 - Construction of a Hirudin HV1  
2    Expression Vector

3

4    An expression vector was designed to enable the  
5    secretion of hirudin to the extracellular medium after  
6    expression in S. cerevisiae. Secretion of hirudin is  
7    desirable as this facilitates production of the protein  
8    with an authentic N-terminus. It also eases  
9    purification, limits intracellular proteolysis, reduces  
10   potential toxic effects on the yeast host and allows  
11   optimal protein folding and formation of native  
12   disulphide bonds. Secretion of hirudin through the  
13   yeast membrane was directed by fusion of hirudin to the  
14   yeast mating type alpha-factor pre-pro-peptide (a  
15   naturally secreted yeast peptide).

16

17   The yeast expression vector pSW6 (Figure 2) is based on  
18   the 2  $\mu$  circle from S. cerevisiae. (pSW6 was deposited  
19   in S. cerevisiae strain BJ2168 at The National  
20   Collection of Industrial and Marine Bacteria Limited,  
21   23 St. Machar Drive, Aberdeen, AB2 1RY, Scotland,  
22   United Kingdom on 23rd October 1990 under Accession No.  
23   NCIMB 40326.) pSW6 is a shuttle vector capable of  
24   replication in both E. coli and S. cerevisiae and  
25   contains an origin of DNA replication for both  
26   organisms, the leu2 gene (a selectable marker for  
27   plasmid maintenance in the yeast host) and the  
28   ampicillin resistant locus for selection of plasmid  
29   maintenance in E. coli. (The DNA sequence of the  
30   vector has been determined; the E. coli sequences are  
31   derived from the E. coli ColE1-based replicon pAT153.)  
32   The full sequence is given as SEQ.ID:4. The ability  
33   to passage this vector through E. coli greatly

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1 facilitates its genetic manipulation and ease of  
2 purification. pSW6 contains an  $\alpha$ -factor  
3 pre-pro-peptide gene fused in-frame to the gene for  
4 epidermal growth factor (EGF). The expression of  
5 this fusion is under the control of an efficient  
6 galactose regulated promoter which contains hybrid DNA  
7 sequences from the S. cerevisiae GAL 1-10 promoter and  
8 the S. cerevisiae phosphoglycerate kinase (PGK)  
9 promoter. Transcription of the EGF gene is terminated  
10 in this vector by the natural yeast PGK terminator.  
11 The EGF gene in pSW6 can be removed by digestion with  
12 restriction endonucleases HindIII and BamHI. This  
13 removes DNA encoding both EGF and 5 amino acids from  
14 the C-terminus of the  $\alpha$ -factor pro-peptide. Genes to  
15 be inserted into the pSW6 expression vector must  
16 therefore have the general composition: HindIII site -  
17  $\alpha$ -factor adaptor - gene- BamHI site.

18

19 To rebuild the DNA encoding the amino acids at the  
20 C-terminal end of the  $\alpha$ -factor pro-peptide and to fuse  
21 this to the synthetic hirudin gene, an oligonucleotide  
22 adapter (5'-AGCTTGGATAAAAGA-3' (top strand, SEQ.ID:5),  
23 5'-TCTTTATCCA-3' (bottom strand, SEQ.ID:6)) containing  
24 a HindIII site and codons encoding the Ser, Leu, Asp,  
25 Lys and Arg from the C-terminal end of the  $\alpha$ -factor  
26 pro-peptide was constructed. The  $\alpha$ -factor adaptor was  
27 ligated to the synthetic HV-1 gene such that the  
28 recombinant gene encoded an in-frame  $\alpha$ -factor  
29 pro-peptide fusion to hirudin. The pUC19 HV-1 plasmid  
30 DNA of Preparation 1 was first cleaved with BspMI and  
31 the overhanging ends were filled using DNA polymerase I  
32 Klenow fragment to create a blunt-ended linear DNA  
33 fragment. The linearised fragment was separated from

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1 uncut plasmid on a 1% low gelling temperature agarose  
2 gel, excised and extracted from the agarose gel matrix,  
3 then further treated with HindIII. The fragment was  
4 then ligated to the alpha-factor adaptor described  
5 above and annealed prior to ligation. The recombinant  
6 ligation products were transformed into competent cells  
7 of E. coli strain HW87 (Preparation 1). Ampicillin  
8 resistant transformants were analysed by preparation of  
9 plasmid DNA, digestion with HindIII and BamHI and  
10 agarose gel electrophoresis. A correct recombinant  
11 plasmid was called pJC80. The  $\alpha$ -factor adaptor -  
12 hirudin sequence was removed from pJC80 on a ca. 223 bp  
13 HindIII-BamHI DNA fragment (SEQ.ID:7). The  
14 fragment was purified on a low gelling temperature  
15 agarose gel and ligated to HindIII and BamHI treated  
16 pSW6 vector DNA. The recombinant ligation products  
17 were transformed into competent cells of E. coli  
18 strain HW87. Ampicillin resistant transformants were  
19 screened by preparation of plasmid DNA, restriction  
20 endonuclease analysis with HindIII and BamHI and  
21 agarose gel electrophoresis. A clone with the correct  
22 electrophoretic pattern pJK1 (Figure 3) was identified.  
23 This plasmid is the basic vector used for wild-type  
24 hirudin HV-1 expression and was used to derive certain  
25 other yeast expression vectors as detailed in the  
26 remaining preparations and examples.

27

28 PREPARATION 3 - Expression of Hirudin Synthetic Gene

29

30 Plasmid expression vector pJK1 of Preparation 2 was  
31 transformed into yeast (S. cerevisiae) strain BJ2168  
32 which has the following genotype:prc-1-407, prb1-1122  
33 pep4-3 leu2 trp1 ura3-52 cir+ using the method of

1 Sherman F. et al (Methods in Yeast Genetics, Cold  
2 Spring Harbor Laboratory, (1986)). All yeast media  
3 was as described by Sherman et al. Using 2 litre shake  
4 flasks, cultures of yeast containing pJK1 were grown  
5 in 1 litre batches of 0.67% synthetic complete medium,  
6 yeast nitrogen base, with amino acids minus leucine and  
7 1% glucose as a carbon source. After overnight growth  
8 at 30°C, the cells were harvested by centrifugation at  
9 3000 rpm for 10 minutes and resuspended in the same  
10 synthetic complete medium except that 1% galactose and  
11 0.2% glucose was used as the carbon source. This  
12 induces gene expression from the hybrid PGK promoter.  
13 Cells were grown in the induction medium for 3 days.  
14 After this period, the supernatant was harvested and  
15 assayed for hirudin activity as described in Example 2,  
16 Section D, below.

17

18 EXAMPLE 1 - Construction of a Hirudin-IEGR-Hirudin  
19 Fusion Gene and a Vector for its Expression

20

21 A factor Xa-cleavable hirudin fusion protein molecule  
22 has been engineered in which two full length hirudin  
23 molecules are joined by the peptide linker sequence  
24 Ile Glu Gly Arg (See SEQ.ID NO:8). The molecule is  
25 designed to be activatable by factor Xa cleavage.  
26 The strategy for construction of the  
27 hirudin-IEGR-hirudin gene is detailed below.

28

29 A gene encoding the hirudin-IEGR-hirudin molecule was  
30 constructed by oligonucleotide directed mutagenesis  
31 and molecular cloning. Mutagenesis was carried out  
32 according to the method of Kunkel et al., Methods in  
33 Enzymology, 154, 367-382 (1987). Host strains are  
34 described below.

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1    E. coli strains

2

3    RZ1032 is a derivative of E. coli that lacks two  
4    enzymes of DNA metabolism: (a) dUTPase (dut), the lack  
5    of which results in a high concentration of  
6    intracellular dUTP, and (b) uracil N-glycosylase (ung)  
7    which is responsible for removing mis-incorporated  
8    uracils from DNA (Kunkel et al., loc. cit.). A  
9    suitable alternative strain is CJ236, available from  
10   Bio-Rad Laboratories, Watford WD1 8RP, United Kingdom.  
11   The principal benefit is that these mutations lead to  
12   a higher frequency of mutants in site directed  
13   mutagenesis. RZ1032 has the following genotype:  
14

15       HfrKL16PO/45[lvsA961-62], dut1, ung1, thi1,  
16       recA, Zbd-279::Tn10, supE44

17

18   JM103 is a standard recipient strain for manipulations  
19   involving M13 based vectors. The genotype of JM103 is  
20   DELTA (lac-pro), thi, supE,strA, endA, sbcB15, hspR4,  
21   F' traD36, proAB, lacIq, lacZDELTAM15. A suitable  
22   commercially available alternative E. coli strain is  
23   E. coli JM109, available from Northumbria Biologicals  
24   Ltd.

25

26   Mutagenesis

27

28   Prior to mutagenesis it was necessary to juxtapose two  
29   adjacent hirudin genes in an M13 mutagenesis vector.  
30   This was accomplished as described below. pJK1  
31   vector DNA of Preparation 2 was prepared and an  
32   aliquot treated with restriction endonucleases BglII  
33   and BamHI, a ca. 466 bp BglII-BamHI DNA fragment from

1   this digestion was gel purified and ligated to BamHI  
2   treated and phosphatased pJC80 vector DNA of  
3   Preparation 2. The recombinant ligation products were  
4   transformed into competent cells of E. coli strain  
5   HW87 (Preparation 1). Ampicillin (100 µg/ml) resistant  
6   clones were analysed by plasmid DNA preparation,  
7   restriction endonuclease digestion and gel  
8   electrophoresis. Clones with inserts in the desired  
9   orientation were identified after digestion with KpnI  
10   which released a DNA fragment of ca. 465bp in length.  
11   (The products of KpnI digestion were analysed on an  
12   agarose gel.) One of the correct clones, pJK002, was  
13   used for the remaining constructions, this vector  
14   contains a ca. 465 bp KpnI DNA fragment which encodes a  
15   C-terminal portion of a first hirudin gene, a  
16   complete α-factor pre-pro-peptide sequence and the  
17   N-terminal portion of a second hirudin gene. In order  
18   to delete the α-factor pre-pro-peptide sequence and to  
19   insert DNA encoding a factor Xa-cleavable amino acid  
20   linker sequence (IEGR), the ca. 465 bp KpnI DNA  
21   fragment was transferred into a bacteriophage  
22   mutagenesis vector M13mp18. Plasmid DNA of pJK002 was  
23   prepared and a portion was digested with KpnI. The ca.  
24   465 bp KpnI DNA fragment from pJK002 was gel purified  
25   and ligated to KpnI treated and phosphatased M13mp18.  
26   The recombinant ligation products were transfected  
27   into competent cells of E. coli strain JM103. Single  
28   stranded DNAs from putative recombinant phage plaques  
29   were prepared and analysed by dideoxy sequence analysis  
30   using the M13 universal sequencing primer (SEQ. ID NO:  
31   10; see below). A clone pGC609 containing the KpnI  
32   fragment in the correct orientation was identified.

33

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1     The  $\alpha$ -factor pre-pro-peptide sequence between the two  
2     hirudin sequences of pGC609 was deleted and the DNA  
3     encoding the Factor Xa-cleavable amino acid linker  
4     (IEGR) inserted by site directed mutagenesis. Single  
5     stranded DNA of pGC609 was prepared from E. coli  
6     strain RZ1032 and was used as a template for  
7     mutagenesis with a 46mer oligonucleotide BB2988:  
8     (5'-CAGTCGGTGTAAACAACTCTTCCTTCGATCTGCAGATATTCTTCTG-3')  
9     (SEQ. ID NO:9). Single stranded DNAs were prepared  
10    from putative mutant plaques and were analysed by  
11    dideoxy DNA sequence analysis using an M13 universal  
12    sequencing primer (United States Biochemical  
13    Corporation. P.O. Box 22400, Cleveland, Ohio 44122.  
14    USA. Product No. 70763 5'-GTTTTCCCAGTCACGAC-3'), (SEQ.  
15    ID NO:10). A correct clone, pGC610, was identified.  
16    To construct the full length hirudin-IEGR-hirudin gene  
17    the central core of the fusion molecule encoded on the  
18    ca. 210 bp KpnI fragment of pGC610 was cloned into the  
19    KpnI site of pJC80 of Preparation 2. Replicative form  
20    DNA of pGC610 was prepared and digested with KpnI. The  
21    ca. 210 bp KpnI DNA fragment encoding the central core  
22    of the hirudin-IEGR-hirudin protein was gel purified  
23    and ligated to KpnI treated and phosphatased pJC80 of  
24    Preparation 2. The recombinant ligation products were  
25    transformed into competent cells of E. coli strain HW87  
26    (Preparation 1). Ampicillin (100  $\mu$ g/ml) resistant  
27    transformants were analysed by preparation of plasmid  
28    DNA, restriction endonuclease digestion with PstI and  
29    agarose gel electrophoresis. A clone with the correct  
30    electrophoretic pattern pDB1 was identified as  
31    containing a ca. 210 bp DNA fragment after PstI  
32    digestion.

33

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1 To create a vector for the expression of the factor  
2 Xa-cleavable hirudin-IEGR-hirudin fusion protein the  
3 gene was cloned into the yeast expression vector pSW6  
4 of Preparation 2. Plasmid DNA of pDB1 was treated  
5 with HindIII and BamHI and the ca. 420 bp HindIII-BamHI  
6 DNA fragment containing the factor Xa-cleavable  
7 hirudin-IEGR-hirudin gene was gel purified and ligated  
8 to HindIII and BamHI treated pSW6 DNA of Preparation 2.  
9 The recombinant ligation products were transformed  
10 into competent cells of E. coli strain HW87.  
11 Ampicillin (100 µg/ml) resistant transformants were  
12 screened by preparation of plasmid DNA, restriction  
13 endonuclease analysis with HindIII and BamHI and  
14 agarose gel electrophoresis. A clone with the correct  
15 electrophoretic pattern pDB2 was identified. pDB2  
16 contained the hirudin-IEGR-hirudin gene fused in frame  
17 to the yeast α-factor pre-pro-peptide sequence. pDB2  
18 plasmid DNA was prepared and used to transform yeast  
19 strain BJ2168 (Preparation 3) according to the method  
20 of Sherman F. et al (Methods in Yeast Genetics, Cold  
21 Spring Harbor Laboratory, New York (1986)).

22  
23 EXAMPLE 2 - Purification of Hirudin and  
24 Hirudin-IEGR-Hirudin

25 The procedure of Preparation 3 was generally followed  
26 for the expression of hirudin and hirudin-IEGR-hirudin  
27 proteins. Hirudin and hirudin-IEGR-hirudin are  
28 purified from yeast culture broth. Cells were first  
29 removed by centrifugation at 3000 rpm for 10 minutes.  
30 The supernatant was then assayed for biological  
31 activity using a chromogenic assay (see below, section  
32 D). Production levels from shake flask cultures  
33 D).

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1    were routinely between 10-15 mg/litre of culture. The  
2    hirudin protein was purified by preparative HPLC  
3    (DYNAMAX (Trade Mark) C18, 300 angstroms). The column  
4    was first equilibrated in 15% acetonitrile, 0.1%  
5    trifluoro acetic acid. Then 2.5-3 mg of hirudin  
6    activity as determined by chromogenic assay (section  
7    D) was loaded onto the column. The protein was  
8    eluted using a 15-40% acetonitrile gradient at 3  
9    ml/minute over 25 min. The purity of the isolated  
10   protein was assessed by analytical HPLC (VYDAC (Trade  
11   Mark) C18 reverse phase), N-terminal sequence analysis  
12   and mono Q FPLC as described below.  
13

14   A. Assessing Purity by Analytical HPLC  
15

16   Samples were analysed on a VYDAC (Trade Mark) C18  
17   column (15 x 0.46cm, particle size 5 micron)  
18   equilibrated with 10% acetonitrile, 0.1% trifluoroacetic  
19   acid (TFA). Purified protein (20 µg) was loaded in  
20   10% acetonitrile, 0.1% TFA. Protein was eluted at a  
21   flow rate of 1ml/minute using an acetonitrile gradient  
22   from 10-40% in 0.1% TFA over 30 minutes. The eluted  
23   protein sample was monitored by absorbance at 280 nm.  
24

25   B. Analysis of Purity by Mono Q FPLC  
26

27   Samples were analysed on a Mono Q FPLC column  
28   (5 x 0.5cm, Pharmacia) equilibrated in 20 mM Tris.HCl  
29   pH 7.5. Approximately 15 µg of lyophilised protein  
30   was reconstituted in 1ml 20mM Tris.HCl pH 7.5 and  
31   loaded onto the column. Protein was eluted using a  
32   gradient of 0-250mM NaCl in 20 mM Tris.HCl buffer  
33   (pH 7.5) at a flow rate of 1ml/minute over 30 minutes.

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1      C.    N-terminal Sequence Analysis

2  
3      N-terminal sequence analysis was performed by  
4      automated Edman degradation using an Applied Biosystems  
5      Protein Sequencer, model 471 A (Applied Biosystems,  
6      Foster City, California).

7  
8      Purified material that was greater than 95% pure, was  
9      dried down in a SPEEDIVAC (trade mark of Savant  
10     Instruments Inc. Hicksville, N.Y. U.S.A.) and  
11     reconstituted in 0.5 ml of 0.9% (w/v) saline for assay.

12  
13     D.    Hirudin Anti-thrombin Chromogenic Activity Assay

14  
15     The ability of hirudin and molecules containing hirudin  
16     to inhibit the thrombin catalysed hydrolysis of the  
17     chromogenic substrate tosyl-Gly-Pro-Arg-p-nitroanilide  
18     (CHROMOZYME TH (trade mark of Boehringer-Mannheim)) was  
19     used as an assay to determine their anti-thrombin  
20     activity.   Protein samples (50 µl) diluted in 0.1M  
21     Tris.HCl pH8.5, 0.15 M NaCl, 0.1% (w/v) PEG 6000 were  
22     mixed with 50 µl human thrombin (Sigma, 0.8 U/ml in the  
23     above buffer) and 50 µl CHROMOZYME TH (2.5mM in water)  
24     in 96 well plates (Costar). The plates were incubated  
25     at room temperature for 30 minutes. The reaction was  
26     terminated by adding 50 µl 0.5 M acetic acid and the  
27     absorbance read at 405 nm using an automatic plate  
28     reader (Dynatech). Quantitation was performed by  
29     comparison with a standard hirudin preparation  
30     (recombinant [Lys-47]-HV-2 purchased from Sigma: Sigma  
31     Chemical Co. Ltd, Fancy Road, Poole, Dorset BH11 7TG,  
32     United Kingdom).

33

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1    EXAMPLE 3 - Cleavage and Activation of Hirudin-IEGR-  
2    Hirudin Fusion Protein

3  
4    Purified hirudin-IEGR-hirudin fusion protein was  
5    incubated with Factor Xa. The reaction was performed  
6    at 37°C in a total volume of 150 µl of 0.1M Tris.HCl  
7    buffer pH 7.8 and contained 2.06 nmol fusion protein  
8    and 0.4 nmol Factor Xa. Analysis of the reaction  
9    mixture by sodium dodecyl sulphate-polyacrylamide gel  
10    electro- phoresis (SDS-PAGE) demonstrated cleavage to  
11    products of a similar size to native hirudin. Reverse  
12    phase HPLC analysis of the cleavage reaction as in  
13    Example 2, section A, demonstrated the appearance of  
14    two new species with retention times (RT) of 17 and 20  
15    minutes compared to 22 minutes for the intact fusion  
16    protein.

17  
18   Measurements of specific activity were made on the  
19   products isolated from a cleavage reaction. Using a  
20   chromogenic assay according to the method of Example  
21   2, section D, to measure hirudin activity in  
22   anti-thrombin units and A 280 nm to determine protein  
23   concentration, the following results were obtained:  
24   product RT 17 min., 6125 U/mg; product RT 20 min.,  
25   5226 U/mg; intact hirudin-IEGR-hirudin, RT 22 min.,  
26   2588 U/mg. Cleavage therefore produces an approximate  
27   2-fold increase in specific activity, with the products  
28   displaying similar values to that recorded for a  
29   recombinant hirudin sample (6600 U/mg) as measured  
30   according to the method of Example 2, section D.

31  
32   Purified cleavage products and the intact fusion  
33   protein were subjected to N-terminal sequence analysis.

1 In each case the sequence obtained was identical to  
2 that of native hirudin (HV1), (VVYTD).

3  
4 It has thus been demonstrated that the  
5 hirudin-IEGR-hirudin fusion protein can be cleaved  
6 by Factor Xa to produce two products with hirudin  
7 activated. Cleavage of the fusion protein is  
8 accompanied by activation as the products of  
9 cleavage have approximately double the specific  
10 activity of the fusion protein.

11  
12 PREPARATION 4 - Isolation of a streptokinase gene

13  
14 Streptokinase is secreted by Lancefield's Group C  
15 streptococci and cloning of the streptokinase gene from  
16 Streptococcus equisimilis strain H46A has been  
17 described (Malke, H. and J.J. Ferretti, P.N.A.S. 81  
18 3557-3561 (1984)). The nucleotide sequence of the  
19 cloned gene has been determined (Malke, H., Roe, B.  
20 and J.J. Ferretti, Gene 34 357-362 (1985)). A gene  
21 encoding streptokinase has been cloned from  
22 S. equisimilis (ATCC 9542 or ATCC 10009) for use in the  
23 current invention. Methods that can be used to  
24 isolate genes are well documented and the procedure  
25 used to isolate the streptokinase gene is summarized in  
26 the following protocol.

27  
28 1. DNA was prepared either from Streptococcus  
29 equisimilis (Lancefield's Group C) ATCC 10009 or from  
30 ATCC 9542 grown in brain-heart infusion medium  
31 (Difco-Bacto Laboratories, PO Box 14B, Central Avenue,  
32 E. Mosely, Surrey KT8 OSE, England) as standing  
33 cultures. Chromosomal DNA was isolated from

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1 approximately 1.5 ml of cells at a density of  $1 \times 10^{11}$   
2 cells/ml. The cells were harvested and washed in 1ml  
3 buffer (0.1M potassium phosphate pH 6.2). The pellet  
4 was resuspended in 400  $\mu$ l of the same buffer and 500  
5 units of mutanolysin (Sigma Chemical Company Ltd, Fancy  
6 Road, Poole, Dorset BH17 7TG, UK) in 100 $\mu$ l volume was  
7 added. This mix was incubated at 37°C for 1 hour. The  
8 cells were harvested by centrifugation and again washed  
9 in buffer. The cells were resuspended in 500 $\mu$ l of a  
10 solution containing 50mM glucose, 10mM EDTA and 25mM  
11 Tris HCl pH 8.0 and incubated at 37°C for approximately  
12 1 hour with the mix being shaken gently to prevent the  
13 cells settling. A 500 $\mu$ l aliquot of a solution  
14 containing 0.4% SDS and proteinase K (100 $\mu$ g/ml) (Sigma  
15 Chemical Company Ltd) was added and the mix was  
16 incubated at 37°C for 1 hour until it became viscous  
17 and clear. The mix was then extracted three times with  
18 phenol equilibrated with TE buffer (10mM Tris HCl, 1mM  
19 EDTA pH 8.0). The aqueous phase was removed into an  
20 eppendorf tube, sodium acetate added to a final  
21 concentration of 0.3M and 2.5 volumes of ethanol added.  
22 The mix was incubated at -70°C for 1 hour to  
23 precipitate the DNA. The DNA was pelleted by  
24 centrifugation, washed with 70% ethanol and then  
25 resuspended in 200  $\mu$ l TE buffer.  
26

27 2. The Polymerase Chain Reaction (PCR) was used to  
28 amplify the streptokinase sequence (Saiki R. et al  
29 Science, 239, 487-491 (1988)). Two primers were  
30 designed based on the published streptokinase  
31 sequences. The primer encoding the antisense strand at  
32 the 3' end of the gene was a 40mer BB1888  
33 (5'GTTCATGGATCCTTATTGTCGTTAGGGTTATCAGGTATA 3'), (SEQ.

1 ID NO:11) which also encoded a BamHI site. The primer  
2 encoding the sense strand at the 5' end of the gene  
3 encoded an EcoRI site in addition to the streptokinase  
4 sequence and was the 40mer BB1887  
5 (5'TCAAGTGAATTGATGAAAAATTACTTATCTTTGGGATGT 3'), (SEQ  
6 ID NO:12). Forty cycles of PCR were performed with the  
7 denaturation step at 95°C for 2 minutes, followed by  
8 annealing of the primers for 3 minutes at 55°C and  
9 extension at 70°C for 4.5 minutes. A sample of the  
10 reaction product was analysed on a 0.8% agarose gel.  
11 A single amplified DNA fragment at c.a. 1.3 kB, which  
12 corresponds to the expected size of the streptokinase  
13 gene, was observed.

14  
15 3. A 30μl sample of the product was digested with the  
16 restriction endonucleases EcoRI and BamHI, analysed on  
17 a low gelling temperature agarose gel and the  
18 c.a. 1.3 kb DNA fragment was isolated from the gel.  
19 The band was extracted from the gel and ligated into  
20 the plasmid pUC19 which had been cleaved with EcoRI and  
21 BamHI to form the plasmid pUC19SK.

22  
23 The entire ca. 1330 bp EcoRI-BamHI fragment from  
24 pUC19SK was sequenced by dideoxy sequence analysis.  
25 To facilitate the sequencing, The EcoRI-BamHI DNA  
26 fragment of pUC19SK was transferred to M13 sequencing  
27 vectors mp18 and mp19 in two halves. A ca. 830 bp  
28 EcoRI-HindIII DNA fragment was separately transferred  
29 into EcoRI and HindIII treated M13mp18 and M13mp19.  
30 The products from these two ligation events were  
31 separately transfected into competent cells of E. coli  
32 host JM103. Single stranded DNA was prepared and used  
33 for dideoxy sequence analysis using the primers listed

1       in SEQ ID NO: 13 and SEQ ID NO: 10. A ca. 490 bp  
2       HindIII-BamHI fragment was gel purified after  
3       treatment of pUC19SK with HindIII and BamHI. This DNA  
4       fragment was separately ligated to M13mp18 and M13mp19  
5       which had been treated with HindIII and BamHI. The  
6       products of these two ligation was transfected into  
7       competent cells of E. coli host JM103. Single stranded  
8       DNA was prepared and used for dideoxy sequence analysis  
9       with the primers shown in SEQ ID NO:13 and SEQ ID  
10      NO: 10. The entire sequence of the EcoRI-BamHI PCR  
11      derived DNA fragment is shown in SEQ ID NO:14.  
12

13      EXAMPLE 4 - Construction of Streptokinase Expression  
14      Vectors

15

16      A number of alternative streptokinase expression  
17      vectors have been constructed for expression in either  
18      yeast S. cerevisiae or E. coli K12.  
19

20      1) Vectors for secretion to the periplasm of E. coli  
21      K12  
22

23      Two vectors were designed to enable the secretion of  
24      streptokinase to the periplasmic space after expression  
25      in E. coli K12. Secretion of streptokinase is  
26      desirable to facilitate production of protein with an  
27      authentic N-terminus, to ease purification, to reduce  
28      potential toxic effects and to limit intracellular  
29      proteolysis. Secretion of streptokinase through  
30      the E. coli cytoplasmic cell membrane was directed by  
31      either the streptokinase signal peptide or the E. coli  
32      major outer membrane protein A (OmpA) signal peptide  
33      (OmpAL).

1    A. Secretion using the streptokinase leader

2  
3    The streptokinase gene of Preparation 4 was  
4    transferred into the E. coli expression vector pGC517  
5    (Figure 4). pGC517 contains the regulatable ptac  
6    promoter, a ribosome binding site and a synthetic  
7    transcriptional terminator. pGC517 was deposited in  
8    E. coli K12 at The National Collection of Industrial  
9    and Marine Bacteria Limited, 23 St. Machar Drive,  
10   Aberdeen, AB2 1RY, Scotland, United Kingdom on 5th  
11   December 1990 under Accession No. NCIMB 40343. Genes  
12   can be cloned into the expression site of pGC517 on  
13   NdeI-BamHI DNA fragments. It was necessary to  
14   engineer a NdeI site into the 5' end of the  
15   streptokinase gene to enable subsequent cloning into  
16   pGC517. The NdeI site was introduced by site-directed  
17   mutagenesis. To construct the vector for the site  
18   directed mutagenesis, plasmid DNA of vector pUC19SK of  
19   Preparation 4 was prepared and digested with EcoRI and  
20   BamHI and the ca. 1.3 Kb EcoRI-BamHI DNA fragment was  
21   gel purified and ligated to M13mp18 treated with  
22   EcoRI and BamHI. Recombinant ligation products were  
23   transfected into competent cells of E. coli strain  
24   JM103 (Example 1). Single stranded DNA was prepared  
25   from the putative recombinant plaques and analysed by  
26   dideoxy sequence analysis using the M13 universal  
27   sequencing primer (SEQ ID NO: 10 of Example 1). One of  
28   the correct recombinant phages was called pGC611.  
29   Single stranded DNA of phage pGC611 was prepared from  
30   E. coli strain RZ1032 (Example 1) and used as a  
31   template for mutagenesis. An NdeI restriction site was  
32   introduced by site-directed mutagenesis at the 5' end  
33   of the streptokinase gene such that the NdeI site

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1 overlapped the streptokinase initiation codon. The  
2 mutagenesis was performed using a 26-mer BB2175  
3 (5'-GATAAGTAATTTTATATGAATTG-3'), (SEQ ID NO:15).  
4 Single stranded DNAs were prepared from putative  
5 mutant plaques and were screened by dideoxy sequence  
6 analysis using the 18mer sequencing primer BB2358  
7 (5'-CATGAGCAGGTCGTGATG-3'), (SEQ ID NO:16) and a  
8 correct clone pGC612 was identified.  
9

10 To construct an expression vector, the streptokinase  
11 gene carrying the newly introduced NdeI site, was  
12 cloned into the pGC517 expression vector. Replicative  
13 form DNA was prepared from pGC612 and was digested  
14 with NdeI and BamHI and the ca. 1.3 kb NdeI-BamHI DNA  
15 fragment was gel purified. This fragment was then  
16 ligated to NdeI and BamHI treated pGC517 DNA. The  
17 recombinant ligation products were transformed into  
18 competent cells of E. coli strain JM103. Ampicillin  
19 (100 µg/ml) resistant transformants were analysed by  
20 plasmid DNA preparation, restriction endonuclease  
21 digestion with BglII and BamHI and agarose gel  
22 electrophoresis. One of the correct clones, pKJ2, was  
23 verified by dideoxy sequence analysis using the  
24 sequencing primer BB2358. This vector contains the  
25 entire streptokinase gene including the sequences  
26 encoding the streptokinase signal peptide leader  
27 region and was used for the expression of streptokinase  
28 in E. coli.  
29

30 B. Secretion using the E. coli OmpA leader  
31

32 As an alternative secretion signal, a DNA sequence  
33 encoding the major outer membrane protein A (OmpA)

1 signal peptide (OmpAL) was fused to the DNA sequence  
2 encoding the mature streptokinase protein; see SEQ ID  
3 NO:17. A DNA fragment encoding streptokinase was  
4 obtained by preparing pUC19SK vector DNA, treating the  
5 DNA with EcoRI and filling-in the overhanging single  
6 stranded DNA ends with DNA polymerase I Klenow  
7 fragment to create a blunt-ended linear DNA fragment.  
8 The fragment was next digested with BamHI and the ca.  
9 1.3 kb blunt-ended-BamHI DNA fragment containing the  
10 streptokinase gene was gel-purified. The DNA sequence  
11 encoding OmpAL is available on an expression vector  
12 pSD15. The pSD15 vector contains a gene encoding an  
13 insulin like growth factor II gene (IGF-II) fused to  
14 the OmpAL signal sequence. pSD15 was deposited in  
15 E. coli K12 at The National Collection of Industrial  
16 and Marine Bacteria Limited, 23 St. Machar Drive,  
17 Aberdeen, AB2 1RY, Scotland, United Kingdom on 5th  
18 December 1990 under Accession No. NCIMB 40342. In  
19 order to use pSD15 as a vector to provide the OmpAL DNA  
20 sequence, pSD15 vector DNA was treated with NheI, the  
21 single stranded DNA overhanging ends were filled-in  
22 with DNA polymerase I Klenow fragment to create a  
23 blunt-ended linear DNA fragment. The linear DNA  
24 fragment was next digested with BamHI which removed  
25 ca. 123 bp from the 3' end of the IGF-II gene in pSD15.  
26 After restriction endonuclease digestion the cleaved  
27 linear DNA fragment was treated with phosphatase, to  
28 prevent recircularisation of any partially cut vector  
29 DNA and was gel purified then ligated to the  
30 blunt-ended-BamHI DNA fragment containing the  
31 streptokinase gene. The ligated mixture was  
32 transformed into competent cells of E. coli strain HW87  
33 (Preparation 1). Ampicillin (100 µg/ml) resistant

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1 recombinants carrying the streptokinase gene were  
2 characterised by preparation of plasmid DNA,  
3 restriction endonuclease analysis with BglII and  
4 HindIII and agarose gel electrophoresis. A construct  
5 of the correct electrophoretic pattern was called pKJ1.  
6 Vector pKJ1 contains the DNA encoding OmpAL and  
7 streptokinase separated by a region of DNA not required  
8 in further constructs. The sequence of the insert DNA  
9 in pKJ1 was confirmed by dideoxy sequence  
10 analysis with a 44-mer oligonucleotide BB58  
11 (5'-AGCTCGTAGACACTCTGCAGTTCGTTGTGGTACCGTGGCTTC-3')  
12 SEQ ID NO:18. In order to create a DNA template for  
13 the deletion loopout mutagenesis of the unwanted DNA  
14 sequence, the BglII to HindIII DNA fragment from pKJ1  
15 was cloned into a vector M13mpl9. pKJ1 vector DNA  
16 was treated with BglII and HindIII to produce a  
17 ca. 1026 bp DNA fragment, which was gel purified and  
18 ligated into the polylinker region of M13mpl9  
19 replicative form DNA treated with BamHI and HindIII.  
20 Ligation products were transfected into competent  
21 cells of E. coli strain JM103. Single stranded DNAs  
22 were prepared from putative recombinant plaques and a  
23 correct clone (pGC600) identified by dideoxy sequence  
24 analysis using the M13 universal sequencing primer (SEQ  
25 ID NO:10, Example 1).

26

27 Mutagenesis on template pGC600 was performed using a  
28 30-mer oligonucleotide mutagenesis primer  
29 BB2658 (5'-ACCGTAGCGCAGGCCATTGCTGGACCTGAG-3') SEQ ID  
30 NO:19. Single stranded DNAs were prepared from  
31 putative mutant plaques and a clone, pGC601, containing  
32 the required deletion was identified using dideoxy  
33 sequence analysis with the M13 universal sequencing

1 primer (SEQ ID NO: 10). pGC601 contains part of the  
2 OmpAL-streptokinase fusion required for the secretion  
3 of streptokinase from this signal peptide in E. coli,  
4 but DNA encoding the C-terminal portion of  
5 streptokinase is absent. In order to reconstruct the  
6 streptokinase gene, replicative form DNA from pGC601  
7 was digested with restriction enzymes NdeI and HindIII  
8 and the ca. 810 bp NdeI-HindIII DNA fragment containing  
9 the DNA sequences encoding OmpAL leader peptide  
10 sequence fused to the N-terminal portion of  
11 streptokinase was gel purified. pJK2 vector DNA was  
12 treated with restriction enzymes NdeI and HindIII  
13 followed by treatment with phosphatase and the ca. 3620  
14 bp NdeI-HindIII vector DNA fragment containing the  
15 essential vector sequences and the C-terminal portion  
16 of the streptokinase gene was gel purified. The  
17 ca. 810 bp NdeI-HindIII (pGC601) and ca. 3620  
18 NdeI-HindIII (pJK2) gel purified DNA fragments were  
19 ligated together and the recombinant ligation products  
20 were transformed into competent cells of E. coli  
21 strain HW1110 (lacIq). The lacIq mutation in this  
22 strain enhances repression of transcription from the  
23 tac promoter. Any other lacIq strain, for example  
24 JM103 could be used instead. The ampicillin resistant  
25 transformants were screened by preparation of plasmid  
26 DNA followed by restriction endonuclease analysis using  
27 NdeI and HindIII. Agarose gel electrophoresis of  
28 digestion products was used to identify a correct clone  
29 which was called pLGC1. The pLGC1 construct was  
30 verified by dideoxy sequence analysis using a 17-mer  
31 oligonucleotide BB2753 (5'-GACACCAACCGTATCAT-3'), (SEQ  
32 ID NO: 20) to sequence through the BamHI site and  
33 primer BB3510 (5'-CACTATCAGTAGCAAAT-3'), (SEQ ID NO:21)

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1 to sequence through the sequence encoding the OmpA  
2 leader.

3

4 2) Intracellular Expression in *E. coli*

5

6 As streptokinase contains no disulphide bonds there is  
7 no requirement for secretion to encourage native  
8 protein folding and although streptokinase is  
9 naturally secreted, intracellular expression offers  
10 several potential advantages such as high yield and  
11 inclusion body formation which may facilitate  
12 purification. As an alternative production route,  
13 an expression vector was designed for intracellular  
14 production of streptokinase in *E. coli*. DNA encoding  
15 the amino acids 2 to 21 of the OmpAL signal peptide  
16 sequence which was fused to mature streptokinase in  
17 pGC601 were deleted by loopout site directed  
18 mutagenesis using single stranded DNA of pGC601  
19 with a 31-mer mutagenesis oligonucleotide  
20 BB3802 (5'-GAAATACTTACATATGATTGCTGGACCTGAG-3'), (SEQ  
21 ID NO:22). In addition to deleting the OmpAL  
22 signal peptide coding sequence, BB3802 fused the  
23 methionine codon (ATG) of the OmpAL signal peptide  
24 sequence to the first codon of mature streptokinase  
25 to create the 5'end of gene encoding a  
26 Methionyl-streptokinase fusion protein (see SEQ ID  
27 NO:23). The ATG codon was used to allow  
28 initiation of translation at the correct position.  
29 Single stranded DNA was prepared from putative mutant  
30 plaques and a clone containing the desired mutation,  
31 pGC602 was identified using dideoxy sequence  
32 analysis with the M13 universal sequencing primer  
33 (SEQ ID NO:10). The C-terminal portion of the

1   streptokinase gene is missing in pGC602. In  
2   order to reconstruct the intact mature streptokinase  
3   coding sequence, replicative form DNA from pGC602 was  
4   digested with restriction enzymes NdeI and HindIII and  
5   the ca. 755 bp NdeI-HindIII DNA fragment encoding  
6   the N-terminal portion of the Methionyl-streptokinase  
7   protein was gel purified and ligated to the gel  
8   purified ca. 3620 bp NdeI-HindIII pLGC2 vector DNA  
9   fragment described in Example 6 below. The recombinant  
10   ligation mixture was transformed into competent cells  
11   of E. coli strain HW1110 (lacIq). Ampicillin  
12   (100 µg/ml) resistant transformants were screened  
13   by plasmid DNA preparation, restriction endonuclease  
14   digestion and agarose gel electrophoresis. A clone,  
15   pGC603, with the correct electrophoretic pattern after  
16   NdeI and HindIII digestion, was identified. Vector  
17   pGC603 was used for the intracellular expression of  
18   Methionyl-streptokinase in E. coli strain HW1110.  
19

20   3) Construction of Expression Vectors for the  
21   Secretion of Streptokinase from the Yeast  
22   S. cerevisiae

23

24   Expression vectors were designed to enable the  
25   secretion of streptokinase to the extracellular  
26   medium after expression in S. cerevisiae. Secretion  
27   of streptokinase is desirable to facilitate production  
28   of protein with an authentic N-terminus, to ease  
29   purification, to limit intracellular proteolysis  
30   and to reduce potential toxic effects on the yeast  
31   host. Secretion of streptokinase through the  
32   yeast membrane was directed by either the natural  
33   streptokinase signal peptide or by fusion of

1 mature streptokinase to the yeast mating type  
2 alpha-factor pre-pro-peptide (a naturally secreted  
3 yeast peptide) see SEQ ID NO:24.

4

5 A) Secretion of Streptokinase using the Streptokinase  
6 Signal Peptide

7

8 The streptokinase gene with its natural signal peptide  
9 was cloned into the yeast expression vector pSW6 to allow its expression in the yeast  
10 S. cerevisiae. Vector DNAs of pKJ2 and pSW6 of Preparation 2 were prepared. Both DNAs were treated  
11 with restriction enzymes BglII and BamHI and the ca. 1420 bp DNA fragment from pKJ2 and the ca. 7460  
12 bp vector DNA fragment from pSW6 were gel purified and ligated together. The recombinant ligation products  
13 were transformed into competent cells of E. coli strain DH5 (supE44, hsdR17, recA1, endA1, gyrA96,  
14 thi-1, relA1), but any other good transforming strain could be used, for example JM109 of Example 1.  
15 Ampicillin (100 µg/ml) resistant transformants were analysed by preparation of plasmid DNA, restriction  
16 endonuclease digestion with BamHI and HindIII and agarose gel electrophoresis. A clone with the correct  
17 electrophoretic pattern psMD1/111 was used for the expression of streptokinase from its own signal peptide  
18 sequence from the yeast S. cerevisiae. Plasmid expression vector psMD1/111 was transferred into yeast  
19 (S. cerevisiae) strain BJ2168 according to the method of Preparation 3.

20

21

22

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1       B)    Secretion of Streptokinase using the pre-pro-  
2       α-Factor Secretion Leader

3  
4       A gene fusion to enable the streptokinase gene of  
5       Preparation 4 to be expressed in yeast and to be  
6       secreted by the yeast mating type α-factor  
7       pre-pro-peptide was designed and constructed using  
8       site-directed mutagenesis and molecular cloning see  
9       SEQ ID NO:24. The construction involved mutagenesis to  
10      create an α-factor-streptokinase fusion gene and  
11      molecular cloning to reconstruct the DNA sequences  
12      encoding the mature streptokinase protein sequence.  
13      Single stranded DNA of pGC600 prepared from  
14      E. coli strain RZ1032 (Example 1) was used as a  
15      mutagenesis template with the 36-mer  
16      oligonucleotide   BB3624  
17      (5'-GTCCAAGCTAACGCTTGGATAAAAGAATTGCTGGACC-3') SEQ ID  
18      NO:25.       Single stranded DNA from putative mutant  
19      plaques were analysed by dideoxy sequence analysis  
20      using the M13 universal sequencing primer (SEQ ID  
21      NO:10) and a mutant clone, pGC614, with the desired  
22      sequence was identified.     In pGC614 the  
23      OmpA-IGFII-Streptokinase signal peptide encoding  
24      sequences of pGC600 have been deleted and the  
25      α-factor linker encoding the C-terminal 5 amino acids  
26      of the α-factor pro-peptide described in Preparation 2  
27      have been inserted. To reconstruct the streptokinase  
28      gene in a yeast expression vector, two stages of  
29      genetic manipulation were required. First the  
30      C-terminal portion of streptokinase was cloned into a  
31      yeast expression vector and this new construct was used  
32      to clone in the N-terminal α-factor-streptokinase  
33      fusion portion of the gene, thus reconstructing a

1 mature streptokinase coding region fused to the  
2  $\alpha$ -factor pre-propeptide gene. Vector DNAs of pKJ2  
3 and pSW6 (Preparation 2) were prepared and digested  
4 with HindIII and BamHI and the ca. 485 bp. DNA  
5 fragment from pKJ2 and the ca. 7750 bp. vector DNA  
6 fragment from pSW6 were gel purified and ligated.  
7 Recombinant ligation products were transformed into  
8 competent cells of E. coli strain DH5. Ampicillin  
9 resistant transformants were screened by preparation  
10 of plasmid DNA, restriction endonuclease digestion with  
11 HindIII and BamHI and agarose gel electrophoresis. A  
12 clone with the correct electrophoretic pattern  
13 pSMD1/119 was isolated. It contains DNA encoding  
14 the C-terminal portion of streptokinase cloned into a  
15 yeast expression vector. The DNA encoding the  
16 N-terminal portion of streptokinase and the alpha-  
17 factor adaptor sequence were next cloned into  
18 pSMD1/119. Replicative form DNA of pGC614 was  
19 prepared and treated with HindIII and ligated to  
20 pSMD1/119 vector DNA which had been treated with  
21 HindIII and phosphatased. The recombinant ligation  
22 products were transformed into competent cells of  
23 E. coli strain DH5. Ampicillin (100  $\mu$ g/ml) resistant  
24 transformants were screened by preparation of plasmid  
25 DNA, restriction endonuclease analysis with DraI and  
26 agarose gel electrophoresis. A clone with the  
27 correct electrophoretic pattern pSMD1/152 gave DraI  
28 digestion products of ca. 4750, 1940, 1520 and 700 bp.  
29 in length. pSMD1/152 was used for the expression and  
30 secretion of streptokinase using the alpha factor  
31 pre-pro-sequence from the yeast S. cerevisiae. Plasmid  
32 expression vector pSMD1/152 was transferred into  
33 yeast (S. cerevisiae) strain BJ2168 according to the  
34 method of Preparation 3.

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1    EXAMPLE 5 - Construction of a Gene Encoding a Core  
2    Streptokinase Protein

3

4    A gene encoding a truncated methionyl streptokinase  
5    molecule (aa 16-383) was designed and constructed by  
6    oligonucleotide directed loopout deletions and  
7    molecular cloning; see SEQ ID NO:26. DNA encoding the  
8    amino acids 2 to 21 of the OmpAL signal sequence, the  
9    DNA encoding IGF-II, the DNA encoding the streptokinase  
10   signal peptide and the first 15 amino acids of the  
11   mature streptokinase protein in pGC600 of Example  
12   4B were deleted by loopout mutagenesis using a  
13   33-mer oligonucleotide BB3862:

14   5'-GAAATACTTACATATGAGCCAATTAGTTGTTAG-3'; SEQ ID NO:27.  
15   Single stranded DNA was prepared from E. coli RZ1032  
16   cells infected with pGC600 and used as the template  
17   for mutagenesis with primer BB3862. Single stranded  
18   DNA was prepared from putative mutant plaques and a  
19   clone pGC604 containing the desired deletion was  
20   identified by dideoxy sequence analysis using the M13  
21   universal sequencing primer (SEQ ID NO:10, Example 1).

22

23   Amino acids 384 to 414 were deleted from  
24   streptokinase by loopout mutagenesis using a  
25   28-mer oligonucleotide BB3904:

26   5'-CCCGGGGATCCTTAGGCTAAATGATAGC-3'; SEQ ID NO:28.  
27   The template for the mutagenesis was single  
28   stranded DNA of M13JK1 of Example 10 containing the  
29   ca. 500 bp HindIII-BamHI DNA fragment encoding the 3'  
30   end of the streptokinase gene from pUC19SK of  
31   Preparation 4. Single stranded DNA from putative  
32   mutant plaques was prepared and a clone pGC605  
33   containing the desired deletion was identified by

1 dideoxy sequence analysis using the M13 universal  
2 sequencing primer (SEQ ID NO:10, Example 1).  
3  
4 The intact core streptokinase molecule was  
5 reconstructed from the two mutated halves by a two  
6 step ligation incorporating the NdeI-HindIII DNA  
7 fragment from pGC604 (containing the DNA encoding the  
8 N-terminal portion of the core streptokinase molecule)  
9 and the HindIII-BamHI DNA fragment from pGC605  
10 (containing the DNA encoding the C-terminal portion of  
11 the core streptokinase molecule) into the vector DNA  
12 pLGC2 of Example 6 below. First the pGC604 DNA was  
13 digested with NdeI and HindIII. A DNA fragment of ca.  
14 710 bp. was gel purified. Vector DNA was prepared  
15 from pLGC2 of Example 6 and treated with NdeI and  
16 HindIII and phosphatased. The linear vector DNA was  
17 gel purified and the two fragments were ligated  
18 together. The recombinant ligation products were  
19 transformed into competent cells of E. coli strain  
20 HW1110. Ampicillin (100 µg/ml) resistant  
21 transformants were screened for the required clone  
22 by preparation of plasmid DNA, restriction  
23 endonuclease analysis with NdeI and HindIII followed  
24 by agarose gel electrophoresis of the digestion  
25 products. One construct with the correct  
26 electrophoretic pattern, pGC617, was identified.  
27  
28 To clone the DNA encoding the C-terminal portion, the  
29 same vector DNA (pLGC2) was treated with HindIII and  
30 BamHI and phosphatased. The pGC605 DNA was treated  
31 with HindIII and BamHI and a ca. 402 bp DNA fragment  
32 was gel purified and ligated into the HindIII and BamHI  
33 treated pLGC2 vector DNA. The recombinant ligation

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1 products were transformed into competent cells of  
2 E. coli strain HW1110. Ampicillin (100 µg/ml)  
3 resistant transformants were screened for the required  
4 clone by preparation of plasmid DNA, restriction  
5 endonuclease analysis with BamHI and HindIII, and  
6 agarose gel electrophoresis of the digestion products.  
7 One construct with the correct electrophoretic pattern  
8 pGC618 was identified. Finally, to reconstruct the  
9 intact core streptokinase gene from the two halves,  
10 pGC617 DNA was treated with HindIII and BamHI and the  
11 ca. 402 bp HindIII-BamHI fragment from pGC618 ligated  
12 to it. pGC618 DNA was digested with HindIII and BamHI  
13 and a ca. 402 bp HindIII-BamHI DNA fragment was gel  
14 purified. pGC617 vector DNA was also treated with  
15 HindIII and BamHI and a ca. 402 bp HindIII-BamHI DNA  
16 fragment from pGC618 was ligated into it. The  
17 ligation products were transformed into competent cells  
18 of E. coli strain HW1110. Ampicillin resistant  
19 transformants were screened by preparation of plasmid  
20 DNA restriction endonuclease analysis with BamHI and  
21 HindIII and agarose gel electrophoresis. A correct  
22 construct, pGC606, was identified.

23

24 EXAMPLE 6 - Construction of Expression vectors  
25 containing a Thrombin Cleavable Streptokinase-  
26 Streptokinase Fusion Gene

27

28 1) Construction of a Secretion Vector for the  
29 Expression of a Thrombin Cleavable Streptokinase-  
30 Streptokinase Fusion

31

32 A gene encoding an OmpAL streptokinase-streptokinase  
33 fusion linked by a thrombin cleavable linker sequence

1 VELQGVVPRG, identical to that at the thrombin  
2 cleavage site in Factor XIII, was designed and  
3 constructed by site directed mutagenesis and  
4 molecular cloning (SEQ ID NO:29). A ca. 1.3 Kb  
5 EcoRI-BamHI DNA fragment containing a streptokinase  
6 gene was gel purified after treatment of the pUC19SK  
7 vector DNA of Preparation 4 with EcoRI and BamHI. A  
8 second DNA fragment encoding a streptokinase gene was  
9 gel purified after BglII and SalI digestion of the  
10 pKJ1 vector DNA of Example 4. A trimolecular ligation  
11 was carried out between these two fragments and  
12 EcoRI and SalI treated pGC517 vector DNA described  
13 in Example 4, section 1A. The recombinant ligation  
14 products were transformed into competent cells of  
15 E. coli strain HW1110 (lacIq). Ampicillin (100 µg/ml)  
16 resistant transformants were screened by preparation  
17 of plasmid DNA, restriction endonuclease analysis with  
18 EcoRI and SalI and agarose gel electrophoresis. A  
19 clone with the correct electrophoretic pattern (pSD93)  
20 was identified. pSD93 contains two tandem copies of the  
21 streptokinase gene separated by a sequence containing  
22 the bacteriophage lambda gene cII ribosome binding  
23 site, and encoding the OmpA signal peptide sequence,  
24 the streptokinase signal peptide sequence and the 5'  
25 part of the IGF-II sequence from pKJ1. To remove this  
26 unwanted intervening sequence and to replace it with  
27 the desired thrombin cleavable linker sequence a  
28 part of pSD93 was transferred into an M13  
29 mutagenesis vector for mutagenesis. Plasmid pSD93 DNA  
30 was digested with HindIII and a ca. 1530 bp DNA  
31 fragment gel purified and ligated to HindIII  
32 treated and phosphatased replicative form M13mp18  
33 DNA. The recombinant ligation products were

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1 transformed into competent cells of E. coli strain  
2 JM103 (Example 1). There are two possible fragment  
3 orientations in such a construction. The  
4 orientation of the clones was determined by preparation  
5 of replicative form DNA and analysing the DNA fragments  
6 produced after XmnI digestion and agarose gel  
7 electrophoresis. One of the clones pSD95 which  
8 contained the fragment in an inverted orientation  
9 (thus preventing translation readthrough by virtue of  
10 fusion to the  $\alpha$ -fragment of  $\beta$ -galactosidase expressed  
11 from the M13 mutagenesis vector) was used for  
12 mutagenesis. Single stranded DNA template was  
13 prepared from pSD95 and used for site directed  
14 mutagenesis. The primer used was a 63-mer  
15 oligonucleotide BB2938:  
16 (5'-GATAACCCTAACGACAAAGTAGAGCTGCAGGGAGTAGTTCCCTCGTGGAAAT-  
17 TGCTGGACCTGAG-3') (SEQ ID NO:30) designed to loop out  
18 the gene cII ribosome binding site, the OmpAL IGF-II  
19 sequence, the streptokinase signal peptide sequence in  
20 pSD95 and to insert a DNA sequence encoding a thrombin  
21 cleavable amino acid sequence. Single stranded DNAs  
22 were prepared from putative mutant plaques and a  
23 correct mutant pGC607 was identified using dideoxy  
24 sequence analysis with primer BB2753 (SEQ ID NO:20) of  
25 Example 4. Replicative form DNA of pGC607 was  
26 prepared and was digested with HindIII and the  
27 ca. 1277 bp HindIII DNA fragment gel purified and  
28 ligated to HindIII treated and phosphatased pLGC1  
29 vector DNA of Example 4. The recombinant ligation  
30 products were transformed into competent cells of  
31 E. coli strain HW1110. Ampicillin resistant  
32 transformants were screened by preparation of plasmid  
33 DNA, restriction endonuclease analysis using HindIII

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1 and agarose gel electrophoresis. This cloning  
2 rebuilds the gene encoding a thrombin cleavable  
3 streptokinase-streptokinase fusion in an expression  
4 vector. A clone (pLGC2) carrying the insert in the  
5 sense orientation was identified by dideoxy sequence  
6 analysis using primers BB2754 (5'-GCTATCGGTGACACCAT-3')  
7 SEQ ID NO:31 and BB3639 (5'-GCTGCAGGGAGTAGTTC-3') SEQ  
8 ID NO:32. pLGC2 was used for the expression of  
9 thrombin cleavable streptokinase-streptokinase fusion  
10 protein in E. coli HW1110.

11

12 2) Construction of a Vector for the Intracellular  
13 Expression of a Thrombin Cleavable Streptokinase-  
14 Streptokinase Fusion Gene.

15

16 A thrombin cleavable methionyl-streptokinase-  
17 streptokinase gene was designed and constructed by  
18 molecular cloning. The gene was constructed from  
19 the methionyl-streptokinase gene of Example 4 and the  
20 HindIII DNA fragment from pGC607 of Example 6,  
21 encoding the C-terminal portion of a first  
22 streptokinase molecule, a thrombin cleavable linker and  
23 an N-terminal portion of a second streptokinase  
24 molecule.

25

26 Replicative form DNA of pGC607 was prepared and was  
27 digested with HindIII and the ca. 1277 bp HindIII DNA  
28 fragment was gel purified and ligated to HindIII  
29 treated and phosphatased pGC603 vector DNA of  
30 Example 4. The recombinant ligation products were  
31 transformed into competent cells of E. coli strain  
32 HW1110 (lacIq). Ampicillin (100 µg/ml) resistant  
33 transformants were screened by preparation of plasmid

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1 DNA, restriction endonuclease analysis with HindIII,  
2 BamHI and PstI and agarose gel electrophoresis of the  
3 digestion products. One construct with the correct  
4 electrophoretic pattern pLGC3, was used for the  
5 intracellular expression of a thrombin cleavable  
6 methionyl-streptokinase-streptokinase fusion protein.

7

8 EXAMPLE 7 - Construction of a Thrombin Cleavable Core  
9 Streptokinase-core Streptokinase Fusion Gene

10 A gene encoding a core methionyl-streptokinase-core  
11 streptokinase fusion linked by a thrombin  
12 cleavable linker sequence VELQGVVPRG, identical to  
13 that at the thrombin cleavage site in Factor XIII, was  
14 designed and constructed by site directed  
15 mutagenesis and molecular cloning see SEQ ID NO:33.  
16 The core streptokinase-core streptokinase fusion gene  
17 was constructed from the core streptokinase monomer  
18 gene of Example 5 and a HindIII DNA fragment  
19 containing the C-terminal portion of a core  
20 streptokinase gene, a thrombin-cleavable linker and an  
21 N-terminal portion of a core streptokinase gene. To  
22 construct the HindIII DNA fragment containing the  
23 appropriate deletions and encoding a thrombin-cleavable  
24 linker, pGC607 of Example 6 was used as a template  
25 for oligonucleotide directed mutagenesis. A 61-mer  
26 oligonucleotide BB3861:

27 (5'-GCTATCATTAGCCGTAGAGCTGCAGGGAGTAGTTCCCTCGTGGAAAGCCAA-  
28 TTAGTTGTTAG-3') SEQ ID NO:34 was used to delete the  
29 streptokinase amino acids 384 to 414, to reconstruct  
30 the thrombin cleavable linker sequence VELQGVVPRG and  
31 to delete the first 15 amino acids of the N-terminus of  
32 streptokinase. Single stranded DNA from putative

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1 mutant plaques was prepared and a correct clone,  
2 pGC608, was identified by dideoxy sequence analysis  
3 using sequencing primer BB2753 of example 8.  
4 Replicative form DNA was prepared from pGC608 and used  
5 in further construction.

6

7 To construct an intact core methionyl-streptokinase-  
8 core-streptokinase fusion, pGC608 DNA was treated  
9 with HindIII and the ca. 1140 bp HindIII DNA  
10 fragment encoding the C-terminal portion of the core  
11 streptokinase molecule, the thrombin cleavable linker  
12 sequence and the N-terminal portion of a core  
13 streptokinase molecule, was gel purified and ligated  
14 to the vector DNA of pGC606 of Example 5 after  
15 treatment with HindIII and phosphatase.. The  
16 recombinant ligation products were transformed into  
17 competent cells of E. coli strain HW1110 (lacIq).  
18 Ampicillin (100 µg/ml) resistant transformants were  
19 analysed by zymography as described in Example 11  
20 below. A correct clone pLGC4, was identified.  
21

22 EXAMPLE 8 - Construction of a Factor Xa-Cleavable  
23 Hirudin-IEGR-Streptokinase Fusion Gene  
24

25 A hirudin-streptokinase fusion has been designed  
26 in which a full length hirudin molecule is joined to  
27 full length streptokinase via an IEGR linker sequence  
28 cleavable by factor Xa; see SEQ ID NO:35. The gene  
29 encoding the hirudin-streptokinase protein was  
30 constructed by site directed mutagenesis and molecular  
31 cloning. In order to juxtapose the hirudin and  
32 streptokinase genes, the DNA fragments encoding  
33 these genes were ligated together. The streptokinase

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1 gene from plasmid pKJ2 of Example 4 was isolated by  
2 gel purification of a ca. 1.4 kbp DNA fragment after  
3 digestion of pKJ2 vector DNA with BglII and BamHI.  
4 This DNA fragment contains all of the streptokinase  
5 gene together with the DNA encoding the streptokinase  
6 signal peptide sequence. This DNA fragment was then  
7 ligated to BamHI treated pJK1 DNA of Preparation 2  
8 which contains the hirudin encoding DNA sequence.  
9 The recombinant ligation products were transformed  
10 into competent cells of E. coli strain HW1110 (lacIq).  
11 Ampicillin (100 µg/ml) resistant transformants were  
12 screened by preparation of plasmid DNA, restriction  
13 endonuclease digestion with HindIII and agarose gel  
14 electrophoresis. There are two possible orientations  
15 for the insert in this cloning event and correct  
16 clones were identified as those which released a  
17 ca. 1080 bp DNA fragment after HindIII digestion as  
18 analysed on agarose gels. One such clone pJK3, which  
19 contains the hirudin gene separated from the  
20 streptokinase gene by the streptokinase signal  
21 peptide sequence, was used in subsequent  
22 manipulations. To create a template for mutagenesis  
23 to delete the intervening sequences and to insert the  
24 DNA encoding the factor Xa cleavable linker sequence,  
25 the hirudin-streptokinase portion of pJK3 was  
26 transferred to a mutagenesis vector M13mp18. Plasmid  
27 DNA of pJK3 was digested with KpnI and BamHI and  
28 the ca. 1490 bp DNA fragment gel purified and ligated  
29 to KpnI and BamHI treated M13mp18 replicative form  
30 DNA. The recombinant ligation products were  
31 transfected into competent cells of E. coli JM103  
32 (Example 1). Single stranded DNA was prepared from  
33 putative recombinant plaques and a correct clone

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1 pSMD1/100 (1.1) was identified. To delete the  
2 streptokinase signal peptide sequence and to insert the  
3 DNA encoding the factor Xa linker sequence single  
4 stranded DNA of pSMD1/100 (1.1) was used as a template  
5 for mutagenesis with a 46-mer oligonucleotide BB3317:  
6 (5'-CACTCAGGTCCAGCAATTCTACCTTCGATCTGCAGATATTCTTCTG-3')  
7 SEQ ID NO:36. Single stranded DNA from putative mutant  
8 plaques were prepared and a mutant pGC615 was  
9 identified by DNA sequence analysis using the  
10 sequencing primer BB3510 (5'-CACTATCAGTAGCAAAT-3') SEQ  
11 ID NO:37. pGC615 contains the C-terminal portion  
12 of the hirudin gene linked to the mature streptokinase  
13 protein coding sequence. In order to reconstruct the  
14 hirudin gene, replicative form DNA of pGC615 was  
15 treated with KpnI and BamHI, the ca. 1320 bp DNA  
16 fragment gel purified and ligated to KpnI and BamHI  
17 treated pJC80 of Preparation 2. The recombinant  
18 ligation products were transformed into competent cells  
19 of E. coli strain DH5 (Example 4). Ampicillin  
20 (100 µg/ml) resistant transformants were screened by  
21 preparation of plasmid DNA, restriction endonuclease  
22 analysis with KpnI, BamHI and HindIII and agarose gel  
23 electrophoresis. A clone with the correct  
24 electrophoretic pattern pSMD1/139 was identified.  
25 This plasmid contains DNA encoding the complete  
26 factor Xa cleavable hirudin-streptokinase fusion  
27 molecule.

28

29 EXAMPLE 9 - Construction of a Vector for the Expression  
30 of a Factor Xa Cleavable Hirudin-IEGR-Streptokinase  
31 Fusion Molecule

32

33 To construct a vector for the expression of the

1 hirudin-IEGR-streptokinase gene, DNA of pSMD1/139 of  
2 Example 8 was treated with HindIII and a ca. 963 bp  
3 DNA fragment encoding part of the yeast alpha factor  
4 secretion signal, all of hirudin, the factor Xa linker  
5 and the 5' part of streptokinase as far as the internal  
6 HindIII site in the streptokinase sequence was gel  
7 purified. This fragment was then ligated to HindIII  
8 treated and phosphatased DNA of pSMD1/119 of Example  
9 4. The recombinant ligation products were transformed  
10 into competent cells of E. coli strain DH5  
11 (Example 4). Ampicillin resistant transformants were  
12 screened by preparation of plasmid DNA, restriction  
13 endonuclease digestion with KpnI and BamHI and  
14 agarose gel electrophoresis. It is possible to  
15 obtain two orientations of the HindIII insert and one  
16 clone in the correct orientation pSMD1/146 was  
17 identified as releasing a ca. 1311 bp fragment after  
18 KpnI and BamHI treatment. pSMD1/146 contains the full  
19 length fusion gene under the control of the  
20 regulatable PAL promoter described in Preparation 2,  
21 and has been designed for the regulated expression  
22 and secretion of the factor Xa-cleavable  
23 hirudin-streptokinase fusion protein. pSMD1/146  
24 plasmid DNA was prepared and used to transform yeast  
25 strain BJ2168 (Preparation 3) according to the method  
26 of Sherman, F. et al., (Methods in Yeast Genetics, Cold  
27 Spring Harbor Laboratory (1986)).

28  
29  
30  
31  
32  
33

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1    EXAMPLE 10 - Construction of a Factor Xa Cleavable  
2    Streptokinase-IEGR-Hirudin Fusion Gene and its  
3    Expression Vector

4

5    A gene encoding a streptokinase-hirudin fusion  
6    protein linked via a Factor Xa cleavage site (IEGR)  
7    was constructed by site-directed mutagenesis and  
8    molecular cloning SEQ ID NO:38. In order to juxtapose  
9    the streptokinase and hirudin genes, DNA fragments  
10   encoding these two gene were ligated together. The  
11   pUC19SK vector DNA of Preparation 4 was prepared and  
12   treated with HindIII and BamHI and the ca. 500 bp DNA  
13   fragment containing the 3' end of the streptokinase  
14   gene was gel purified. This fragment was ligated to  
15   M13mp19 replicative form DNA treated with HindIII and  
16   BamHI. The recombinant ligation mixture was  
17   transfected into competent cells of E. coli strain  
18   JM103 (Example 1). Single stranded DNA was prepared  
19   from putative recombinant plaques and the required  
20   clone M13JK1 identified by dideoxy sequence analysis  
21   using the M13 universal sequencing primer (SEQ ID  
22   NO:10, Example 1). M13JK1 contains the C-terminal  
23   portion of the streptokinase gene. The  $\alpha$ -factor  
24   hirudin gene was then cloned into M13JK1 to  
25   juxtapose both sequences. Plasmid DNA of pJK1 of  
26   Preparation 2 was digested with BglII and BamHI and  
27   a ca. 465bp DNA fragment encoding the  $\alpha$ -factor hirudin  
28   fusion was gel purified. This DNA fragment was then  
29   ligated to BamHI treated replicative form DNA of  
30   M13JK1. The recombinant ligation products were  
31   transfected into competent cells of E. coli strain  
32   JM103. Single stranded DNA from putative recombinant  
33   plaques were prepared and a correct clone

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1 SMD1/100.3 identified by dideoxy sequence analysis  
2 using M13 universal sequencing primer (SEQ ID NO:10,  
3 Example 1. SMD1/100.3 contains the C-terminal portion  
4 of the streptokinase gene and the complete hirudin  
5 gene separated by the  $\alpha$ -factor encoding sequence  
6 described in Preparation 2. In order to delete this  
7 sequence and replace it with a factor Xa-cleavable  
8 linker sequence, SMD1/100.3 was used as a template  
9 for site-directed mutagenesis. Single stranded DNA  
10 of SMD1/100.3 was prepared and used for mutagenesis  
11 using a 47-mer mutagenesis primer BB3318:  
12 (5'-TCGGTGTAAACAACTCTTCTACCTTCGATTTGTCGTTAGGGTTATC-3")  
13 (SEQ ID NO:40). Single stranded DNA from putative  
14 mutant plaques were prepared and the required mutation  
15 pGC616 identified by dideoxy sequence analysis  
16 using the sequencing primer BB2018:  
17 (5'-GCGGCTTGCGGTACCTTCACCAGTGACACATTGG-3') (SEQ ID  
18 NO:2). pGC616 contains an additional mutation  
19 inadvertently introduced by the mutagenesis procedure.  
20 This was corrected by a further mutagenic step. Single  
21 stranded DNA of pGC616 was prepared and used as a  
22 template for mutagenesis with a 21-mer  
23 oligonucleotide BB3623 (5'-GTGTAAACAACTCTACCTTCG-3')  
24 (SEQ ID NO:40). Single stranded DNA from putative  
25 mutant plaques was prepared and a correct clone pGC620  
26 identified by dideoxy sequence analysis with the  
27 sequencing primer BB2018 (SEQ ID NO:2). pGC620  
28 contains the C-terminal portion of the streptokinase  
29 gene and the complete hirudin gene fused via DNA  
30 encoding a factor Xa-cleavable linker. The intact  
31 factor Xa-cleavable streptokinase-hirudin fusion gene  
32 was reconstructed in two steps. The C-terminal  
33 streptokinase-hirudin sequence from pGC620 was cloned

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1 into the yeast expression vector pSW6 of  
2 Preparation 2 and then the N-terminal portion of  
3 streptokinase was cloned into the new vector to  
4 create the full length streptokinase-hirudin fusion  
5 gene.

6

7 Replicative form DNA of pGC620 was treated with HindIII  
8 and BamHI and a ca. 710 bp HindIII-BamHI DNA  
9 fragment encoding the 3' end of streptokinase, the  
10 intervening factor Xa-cleavable linker DNA sequence and  
11 all of the hirudin gene was gel purified. This  
12 ca. 710 bp DNA fragment was ligated to pSW6 of  
13 Preparation 2 digested with HindIII and BamHI. The  
14 recombinant ligation products were transformed into  
15 competent cells of E. coli strain DH5 (Example 4).  
16 Ampicillin (100 µg/ml) resistant transformants were  
17 screened by preparation of plasmid DNA, restriction  
18 endonuclease analysis using HindIII and BamHI  
19 and agarose gel electrophoresis. A clone with the  
20 correct electrophoretic pattern pSMD1/143 was  
21 identified. The intact fusion gene was then  
22 constructed by cloning the N-terminal portion of  
23 α-factor-streptokinase into pSMD1/143. Replicative  
24 form DNA of pGC614 of Example 4 was treated with  
25 HindIII and the ca. 750 bp DNA fragment containing the  
26 N-terminal portion of α-factor-streptokinase gel  
27 purified and ligated to HindIII treated and  
28 phosphatased pSMD1/143 vector DNA. The recombinant  
29 ligation products were transformed into competent cells  
30 of E. coli strain DH5. Ampicillin (100 µg/ml)  
31 resistant transformants were screened by preparation of  
32 plasmid DNA, restriction endonuclease digestion with  
33 DraI and agarose gel electrophoresis. A clone in the

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1 correct orientation pSMD1/159 was identified as giving  
2 rise to 4 fragments of sizes of about 4750 bp,  
3 2140 bp, 1526 bp, and 692 bp after DraI digestion.  
4 pSMD1/159 was used for the expression of the factor  
5 Xa-cleavable streptokinase-hirudin fusion protein.  
6 pSMD1/159 plasmid DNA was prepared and used to  
7 transform yeast strain BJ2168 (Preparation 5) according  
8 to the method of Sherman, F. et al., (Methods in  
9 Yeast Genetics, Cold Spring Harbour Laboratory (1986)).

10

11 EXAMPLE 11 - Expression of Monomer Streptokinase  
12 Constructs

13

14 Expression

15

16 Competent cells of E. coli strain JM103 (Example 1)  
17 were transformed with DNA of the streptokinase  
18 expression vectors of Examples 4, 5, 6 and 7. The  
19 lacIq gene in the expression host is desirable to  
20 repress transcription from the tac promoter used in all  
21 of the E. coli expression constructs. All media for  
22 the growth of recombinant E. coli strains were as  
23 described in Maniatis et al. Using 1 litre shake  
24 flasks, cultures of recombinant E. coli containing  
25 streptokinase expression vectors were grown in 250 ml  
26 batches of 2TY medium containing 100 µg/ml of  
27 carbenicillin at 37°C in an orbital shaker. The  
28 optical density of the cultures were monitored at  
29 600 nm. When the culture reached an OD 600 nm of 0.5,  
30 expression from the tac promoter was induced by  
31 the addition of isopropyl-β-D-thiogalactoside (IPTG) to  
32 a final concentration of 1 mM. After growth for 30 to  
33 240 min the cells were harvested by centrifugation.

1      SDS-PAGE Separation

2

3      The ability of the recombinant E. coli cells to express  
4      streptokinase was assayed using zymography. The  
5      quantity and molecular weight of streptokinase  
6      activity of an E. coli culture was estimated by the  
7      following protocol. A 1 ml aliquot of the culture  
8      was removed, the cells were harvested by  
9      centrifugation (14 000xg) for 5 mins and resuspended  
10     in 200 µl of loading buffer (125 mM Tris.HCl pH 6.8,  
11     10% glycerol (w/v), 0.01% (w/v) bromophenol  
12     blue, 1% (v/v) 2-mercaptoethanol, 6M urea). An  
13     aliquot of this mixture was applied to an SDS-PAGE gel  
14     and the protein separated by electrophoresis. The  
15     quantity of protein loaded onto the gel was varied  
16     by altering the size of the aliquot according to the  
17     optical density of the culture upon harvesting.  
18     Generally, 10 µl of the mixture from a culture of OD  
19     600 nm of 1.0 was used for each lane.

20

21      Zymography

22

23      After electrophoresis the polyacrylamide gel was washed  
24      in 2% (w/v) Triton X-100 (3x20 mins) followed by  
25      water washes (3x20 mins) to remove the SDS and allow  
26      renaturation of the streptokinase molecule.

27

28      The washed SDS-PAGE gel was then overlayed with an  
29      agarose mixture prepared as follows. 200 mg of agarose  
30      was dissolved in 18 ml distilled and deionised water  
31      (dH<sub>2</sub>O) and allowed to cool to 55-60°C. To this 200 mg  
32      of MARVEL (trade mark of Premier Brands, U.K. Ltd. P.O.  
33      Box 171, Birmingham, B30 2NA, U.K.) (casein) dissolved

1   in 2 ml of dH<sub>2</sub>O, 1 ml of 1M Tris.HCl pH 8.0 and 600  
2   μl of 5M NaCl were added. Just before pouring over  
3   the washed SDS-PAGE gel, 700 μl of plasminogen at  
4   300 μg/ml (Sigma P-7397 10 mg/ml in 50 mM Tris.HCl pH  
5   7.5) was added and mixed thoroughly. The mixture was  
6   poured over the gel and once set was incubated at  
7   37°C for 2 hours when it could be inspected.  
8   Plasminogen activator activity (streptokinase  
9   activity) was detected by plasmin digestion of  
10   the opaque casein containing overlay which produced  
11   clear zones. The position of the zones on the gel  
12   was directly related to the size of the active  
13   molecules.

14  
15   The recombinant *E. coli* JM103 strains containing  
16   monomer streptokinase expression vectors pKJ2 of  
17   Example 4 and pLGC1 of Example 4 both expressed  
18   streptokinase activity with a molecular weight  
19   of approximately 47 kDa (Figure 5).

20  
21   EXAMPLE 12 - Expression of a Thrombin Cleavable  
22   Streptokinase-Streptokinase Fusion Protein.

23  
24   A recombinant *E. coli* HW1110 (*lacIq*) strain (Example  
25   1) containing pLGC2 of Example 6, the thrombin  
26   cleavable streptokinase- streptokinase fusion gene,  
27   was expressed and analysed according to the  
28   expression and zymography protocols of Example 11.  
29   The *E. coli* JM103/pLGC2 strain expressed streptokinase  
30   activities of several molecular weights, predominantly  
31   of 110 kDa and 47 kDa (Figure 5). Cleavage analysis is  
32   described in Example 13 below.

33

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1    EXAMPLE 13 - Cleavage of the Thrombin Cleavable  
2    Streptokinase-streptokinase Fusion Protein by Thrombin

3  
4    Using 1 litre shake flasks, a 3 litre culture  
5    of E. coli JM103 (Example 1) containing pLGC2 of  
6    Example 6 was grown in 500 ml batches in 2TY medium  
7    containing 100 mcg/ml carbenicillin at 37°C with  
8    vigorous shaking in an orbital shaker. When the  
9    optical density of the cultures reached an O.D.  
10   600 nm of 0.5 the expression of the streptokinase-  
11   streptokinase fusion protein was induced by the  
12   addition of IPTG to a final concentration of 1 mM. The  
13   cultures were incubated at 37°C with vigorous shaking  
14   for a further 4 hours when they were harvested by  
15   centrifugation at 8,000 r.p.m. for 10 mins. The cells  
16   were resuspended in 10 ml of ice cold TS buffer  
17   (10 mM Tris.HCl pH 7.5, 20% (w/v) sucrose). 348 µl  
18   of 0.5 M EDTA was added and the mixture incubated on  
19   ice for 10 mins. The cells were harvested by  
20   centrifugation at 8,000 r.p.m. for 5 min at 4°C and  
21   the supernatant discarded. The cells were resuspended  
22   in 6.25 ml of ice cold sterile H<sub>2</sub>O and incubated on  
23   ice for 10 min. The cells were harvested by  
24   centrifugation at 8,000 rpm. for 5 min at 15,000 g for  
25   30 min at 4°C and the supernatant discarded. The cells  
26   were resuspended in 48 ml of ARG buffer (20 mM Tris.HCl  
27   pH 7.5, 10 mM MgCl<sub>2</sub>, 10mM 2-b-mercaptoethanol, 0.5 mM  
28   phenylmethyl sulphonyl fluoride, .12 mM N-tosyl-L-phenylalanine chloromethyl ketone) and  
29   sonicated on ice (6 x 30 sec. bursts on maximum power,  
30   MSE SONIPREP 150 (trade mark)). The cell sonicate was  
31   centrifuged at 15,000 g for 30 min at 4°C. The  
32   supernatant was decanted and assayed for streptokinase  
33

1 activity using the S2251 (KabiVitrum Ltd, KabiVitrum  
2 House, Riverside Way, Uxbridge, Middlesex, UB8 2YF, UK)  
3 chromogenic assay for the streptokinase activation of  
4 plasminogen. S2251 is a specific tripeptide  
5 chromogenic substrate for plasmin. 25  $\mu$ l of 0.1 M  
6 Tris.HCl pH 8.0 was placed in wells 2 to 12 of 96  
7 well plates. Aliquots of the sample (25  $\mu$ l) were  
8 placed in wells 1 and 2, and two-fold dilutions made by  
9 mixing and pipetting from wells 2 to 3, 3 to 4 and so  
10 on to well 11. A 100  $\mu$ l aliquot of a  
11 plasminogen/S2251 mixture (40  $\mu$ l plasminogen 300  $\mu$ g/ml,  
12 220  $\mu$ l S2251 1 mg/ml, 1.04 ml 0.1 M Tris.HCl pH  
13 7.5) was added to each well and the plate incubated  
14 at 37°C for 30 min. The reaction was terminated by  
15 the addition of 50  $\mu$ l of 0.5 M acetic acid. The  
16 absorbance was read at 405 nm using an automatic plate  
17 reader. Quantification was performed by comparison  
18 with a standard streptokinase preparation. The  
19 analysis showed that the supernatant contained  
20 approximately 2560 u/ml of streptokinase activity.

21

22 Solid ammonium sulphate was slowly added to the  
23 supernatant to 15% saturation (4.03 g) and allowed to  
24 dissolve for 15 min at room temperature. The mixture  
25 was then centrifuged for 30 min at 15,000 g at room  
26 temperature. The supernatant was decanted and  
27 additional solid ammonium sulphate was added to 40%  
28 saturation (7.27 g), and allowed to dissolve. The  
29 mixture was centrifuged for 30 min at 15,000 g at room  
30 temperature and the supernatant discarded. The  
31 pelleted protein (the 15-40% cut), was resuspended in  
32 10 ml of ARG buffer. A portion of the 15-40% cut was  
33 assayed using the S2251 chromogenic assay and was found  
34 to contain 18,432 u/ml of streptokinase activity.

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1   The ability of thrombin to cleave the streptokinase-  
2   streptokinase fusion protein at the thrombin cleavable  
3   linker was assessed by an in vitro cleavage assay and  
4   zymography. A 5  $\mu$ l aliquot of the 15-40% cut was  
5   mixed with 45  $\mu$ l of ARG buffer to dilute the  
6   protein ten-fold. 10  $\mu$ l of this mixture was  
7   incubated with 5 u/ml of thrombin in a final volume  
8   of 50  $\mu$ l at 37°C for 14 hours. Aliquots (10  $\mu$ l) of  
9   the thrombin cleavage reactions were analysed by  
10   zymography according to the method of Example 11. The  
11   results are shown in Figure 6. The  
12   streptokinase-streptokinase fusion protein (Mr 110  
13   kDa), was quantitatively cleaved whilst the lower  
14   molecular weight streptokinase activity (Mr 47 kDa) was  
15   not cleaved by thrombin. Thus the streptokinase-  
16   streptokinase fusion protein is cleavable by  
17   thrombin.

18

19   **EXAMPLE 14 - Expression of a Factor Xa Cleavable**  
20   **Streptokinase-IEGR-hirudin Fusion Gene**

21

22   Plasmid expression vector pSMD1/159 of Example 10 was  
23   transferred into yeast (S. cerevisiae) strain BJ2168  
24   according to the method of Preparation 3. Using  
25   500 ml shake flasks, cultures of yeast containing  
26   pSMD1/159 were grown in 100 ml batches of 0.67%  
27   synthetic complete medium yeast nitrogen base, with  
28   amino acids minus leucine and 1% glucose as a carbon  
29   source. After overnight growth at 30°C, the cells  
30   were harvested by centrifugation at 3,000 rpm for  
31   10 min and resuspended in the same synthetic complete  
32   medium except having 1% galactose and 0.2% glucose as  
33   the carbon source and the addition of sodium phosphate

1 (to 50 mM) pH 7.2. This induces the expression of  
2 the streptokinase-hirudin fusion gene from the hybrid  
3 PGK promoter. Cells were grown in the induction  
4 medium for 3 days. After this period, the supernatant  
5 was harvested by centrifugation. The broth was  
6 assayed for both streptokinase activity according to  
7 the S2251 assay procedure of Example 13 and hirudin  
8 activity according to the thrombin inhibition assay  
9 of Example 2. Both activities were detected and  
10 secreted to the medium.

11

12 EXAMPLE 15 - Expression of a Factor Xa Cleavable  
13 Hirudin-IEGR-Streptokinase Fusion Gene

14

15 Plasmid expression vector pSMD1/146 of Example 9  
16 was transferred into yeast (S. cerevisiae) strain  
17 BJ2168 according to the method of Preparation 3. The  
18 culture was incubated, expressed, harvested and the  
19 hirudin and streptokinase activities assayed according  
20 to the methods of Examples 2 and 13. Both  
21 streptokinase and hirudin activities were detected and  
22 secreted to the medium.

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SEQUENCE LISTINGS

SEQ. ID NO:1

SEQUENCE TYPE: nucleotide with corresponding protein  
 SEQUENCE LENGTH: 201 base pairs  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SOURCE: synthetic  
 FEATURES: hirudin type HV-1 gene  
              from 195 to 201 bp non-translated stop codons

SEQUENCE:

GTT	GTT	TAC	ACC	GAC	TGT	ACT	GAA	TCC	GGA	CAA	AAC	CTG	TGT	TTG	45
CAA	CAA	ATG	TGG	CTG	ACA	TGA	CTT	AGG	CCT	GTT	TTG	GAC	ACA	AAC	
Val	Val	Tyr	Thr	Asp	Cys	Thr	Glu	Ser	Gly	Gln	Asn	Leu	Cys	Leu	
					5					10					15
TGT	GAG	GGT	TCT	AAC	GTC	TGT	GGT	CAG	GGT	AAC	AAA	TGC	ATC	CTG	90
ACA	CTC	CCA	AGA	TTG	CAG	ACA	CCA	GTC	CCA	TTG	TTT	ACG	TAG	GAC	
Cys	Glu	Gly	Ser	Asn	Val	Cys	Gly	Gln	Gly	Asn	Lys	Cys	Ile	Leu	
					20					25					30
GGT	TCC	GAC	GGT	GAA	AAG	AAC	CAA	TGT	GTC	ACT	GGT	GAA	GGT	ACC	135
CCA	AGG	CTG	CCA	CTT	TTC	TTG	GTT	ACA	CAG	TGA	CCA	CTT	CCA	TGG	
Gly	Ser	Asp	Gly	Glu	Lys	Asn	Gln	Cys	Val	Thr	Gly	Glu	Gly	Thr	
					35					40					45
CCA	AAG	CCG	CAG	TCC	CAC	AAC	GAT	GGA	GAT	TTC	GAA	GAA	ATC	CCA	180
GGT	TTC	GGC	GTC	AGG	GTG	TTG	CTA	CCT	CTA	AAG	CTT	CTT	TAG	GGT	
Pro	Lys	Pro	Gln	Ser	His	Asn	Asp	Gly	Asp	Phe	Glu	Glu	Ile	Pro	
					50					55					60
GAA	GAA	TAT	CTG	CAG	TAATAG										201
CTT	CTT	ATA	GAC	GTC	ATTATC										
Glu	Glu	Tyr	Leu	Gln											
					65										

\*\*\*\*\* END OF SEQ ID NO: 1 \*\*\*\*\*

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SEQ. ID NO:2

SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 223 base pairs  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SOURCE: synthetic  
 FEATURES: oligomers designed for construction of  
 synthetic type HV-1 gene.  
 SEQUENCE:

BB2011	BB2013					
AGCTTACCTG	CCATGGTTGT	TTACACCGAC	TGTACTGAAT	C	CGGACAAAAA	50
ATGGAC	GGTACCAACA	AATGTGGCTG	ACATGACTTA	GGCCTGTT	TT	
BB2012						
BB2015						
CCTGTGTTTG       TGTGAGGGTT       CTAACGTC       TG       TGGTCAGGGT       AACAAATGCA       100						
GGACACAAAC       ACACTCCCAA       GATTGCAGACACC       AG       TCCA       TTGTTTACGT						
BB2014						
BB2017						
TCCTGGGTTC       CGACGGTG       AA       AAGAACCAAT       GTGTCACTGG       TGAAGGTACC       150						
AGGACCCAAG       GCTGCCACTTTCT       T       GGTTA       CACAGTGACC       ACTTCCATGG						
BB2016						
BB2018						
BB2019						
CCA       AAGCCGC       AGTCCCACAA       CGATGGAGAT       TTCGAAGAAA       TC       191						
GGTTTCGGCG       TCAGGGTGTT       GCTACCTCTA       AAGCTTCTTT       AGGGTCTTC						
BB2020						
BB2021						
CCAGAAGAATATCTGCAG       TAATAGGGAT       CCG       223						
TTATAGACGTC       ATTATCCCTA       GGCTTAA						
BB2022						

\*\*\*\*\* END OF SEQ ID NO: 2 \*\*\*\*\*

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70

SEQ. ID NO:3

SEQUENCE TYPE: nucleotide

SEQUENCE LENGTH: 19 base pairs

FEATURES: Universal sequencing primer complementary  
to the universal primer region of pUC19.

SEQUENCE:

CAGGGTTTTC CCAGTCACG

19

\*\*\*\*\* END OF SEQ ID NO: 3 \*\*\*\*\*

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SEQ ID NO: 4

SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 7859 base pairs  
 STRANDEDNESS: single  
 TOPOLOGY: circular  
 SOURCE: experimental  
 FEATURES: Sequence of plasmid psw6  
 SEQUENCE:

TTCCCATGTC	TCTACTGGTG	GTGGTGCTTC	TTTGGAAATT	TTGGAAGGTA	50
AGGAATTGCC	AGGTGTTGCT	TTCTTATCCG	AAAAGAAATA	AATTGAATTG	100
AATTGAAATC	GATAGATCAA	TTTTTTTCTT	TTCTCTTCC	CCATCCTTTA	150
CGCTAAAATA	ATAGTTTATT	TTATTTTTG	AATATTTTTT	ATTTATATAC	200
GTATATATAG	ACTATTATTT	ACTTTTAATA	GATTATTAAG	ATTTTTTATTA	250
AAAAAAAATT	CGTCCTCTT	TTTAATGCCT	TTTATGCAGT	TTTTTTTCC	300
CATTCGATAT	TTCTATGTTC	GGGTTTCAGC	GTATTTTAAG	TTTAATAACT	350
CGAAAATTCT	GCCTTTCGAA	AAAGCTCTGC	ATTAATGAAT	CGGCCAACGC	400
GCGGGGAGAG	GCCTTTCGCG	TATTGGGCGC	TCTTCGCGTT	CCTCGCTCAC	450
TGACTCGCTG	CGCTCGGTG	TTCGGCTGCG	GCGAGCGTA	TCAGCTCACT	500
CAAAGGCGGT	AATACGGTTA	TCCACAGAAT	CAGGGGATAA	CGCAGGAAAG	550
AACATGTGAG	CAAAAGGCCA	GCAAAAGGCC	AGGAACCCTA	AAAAGGCCGC	600
GTTGCTGGCG	TTTTTCCATA	GGCTCCGCC	CCCTGACGAG	CATCACAAAA	650
ATCGACGCTC	AAGTCAGAGG	TGGCGAAACC	CGACAGGACT	ATAAAGATAC	700
CAGGGCTTTC	CCCCTGGAAAG	CTCCCTCGTG	CGCTCTCCTG	TTCCGACCCCT	750
GCCGCTTACC	GGATACCTGT	CCGCCTTTCT	CCCTTCGGGA	AGCGTGGCGC	800
TTTCTCATAG	CTCACCGCTG	AGGTATCTCA	GTTCGGTGTA	GGTCGTTCGC	850
TCCAAGCTGG	GCTGTGTGCA	CGAACCCCCC	GTTCAGCCCG	ACCGCTGCGC	900
CTTATCCGGT	AACTATCGTC	TTGAGTCCAA	CCCGGTAAGA	CACGACTTAT	950
CGCCACTGGC	AGCAGCCACT	GGTAACAGGA	TTAGCAGAGC	GAGGTATGTA	1000
GGCGGTGCTA	CAGAGTTCTT	GAAGTGGTGG	CCTAACTACG	GCTACACTAG	1050
AAGGACAGTA	TTTGGTATCT	GCGCTCTGCT	GAAGCCAGTT	ACCTTCGGAA	1100
AAAGAGTTGG	TAGCTCTTGA	TCCGGCAAAC	AAACCACCGC	TGGTAGCGGT	1150
GGTTTTTTTG	TTTGCAAGCA	GCAGATTACG	CGCAGAAAAAA	AAGGATCTCA	1200
AGAAGATCCT	TTGATCTTTT	CTACGGGTC	TGACGCTCAG	TGGAACGAAA	1250
ACTCACGTTA	AGGGATTTCG	GTCATGAGAT	TATCAAAAG	GATCTTCACC	1300
TAGATCCTTT	TAAATTAAAA	ATGAAGTTT	AAATCAATCT	AAAGTATATA	1350
TGAGTAAACT	TGGTCTGACA	GTTACCAATG	CTTAATCAGT	GAGGCACCTA	1400
TCTCAGCGAT	CTGTCCTATT	CGTTCATCCA	TAGTTGCC	ACTCCCCGTC	1450
GTGTAGATAA	CTACGATACG	GGAGGGCTTA	CCATCTGGCC	CCAGTGTGTC	1500
AATGATACCG	CGAGACCCAC	GCTCACCGGC	TCCAGATT	TCAGCAATAA	1550
ACCAGCCAGC	CGGAAGGGCC	GAGCGCAGAA	GTGGTCC	AACTTTATCC	1600
GCCTCCATCC	AGTCTATTAA	TTGTTGCCGG	GAAGCTAGAG	TAAGTAGTTC	1650
GCCAGTTAAT	AGTTTGC	ACGTTGTTGC	CATTGCTACA	GGCATCGTGG	1700
TGTCACGCTC	GTCGTTGGT	ATGGCTTCAT	TCAGCTCCGG	TTCCCAACGA	1750
TCAAGGCGAG	TTACATGATC	CCCCATGTTG	TGCAAAAAG	CGGTTAGCTC	1800
CTTCGGTCCT	CCGATCGTTG	TCAGAAAGTAA	GTTGGCCGCA	GTGTTATCAC	1850
TCATGGTTAT	GGCAGCACTG	CATAATTCTC	TTACTGTCA	GCCATCCGTA	1900
AGATGCTTTT	CTGTGACTGG	TGAGTACTCA	ACCAAGTCAT	TCTGAGAATA	1950

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GTGTATGCGG	CGACCGAGTT	GCTCTTGC	GGCGTCAACA	CGGGATAATA	2000
CCCGGCCACA	TAGCAGAACT	TTAAAAGTGC	TCATCATTGG	AAAACGTTCT	2050
TCGGGGCGAA	AACTCTCAAG	GATCTTACCG	CTGTTGAGAT	CCAGTTCGAT	2100
GTAACCCACT	CGTCACCCA	ACTGATCTTC	AGCATCTTT	ACTTTCACCA	2150
GCGTTCTGG	GTGAGCAAAA	ACAGGAAGGC	AAAATGCCGC	AAAAAAGGGA	2200
ATAAGGGCGA	CACGGAAATG	TTGAATACTC	ATACTCTTCC	TTTTTCAATA	2250
TTATTGAAGC	ATTTATCAGG	GTATTGTC	CATGAGCGGA	TACATATTG	2300
AATGTATTAA	GAAAAATAAA	CAAATAGGGG	TTCCGCGCAC	ATTTCCCCGA	2350
AAAGTGCCAC	CTGACGTCTA	AGAAACCATT	ATTATCATGA	CATTAACCTA	2400
AAAAAAATAGG	CGTATCACGA	GGCCCTTTCG	TCTTCAAGAA	TTCTGAACCA	2450
GTCCTAAAC	GAGTAAATAG	GACCGGCAAT	TCTTCAAGCA	ATAAACAGGA	2500
ATACCAATT	TTAAAAGATA	ACTTAGTCAG	ATCGTACAAT	AAAGCTAGCT	2550
TTGAAGAAAA	ATGCGCCTTA	TTCAATCTT	GCTAAAAAA	ATGGCCAAA	2600
ATCTCACATT	GGAAAGACATT	TGATGACCTC	ATTTCTTCA	ATGAAGGGCC	2650
TAACGGAGTT	GACTAATGTT	GTGGGAAATT	GGAGCGATAA	GCGTGCTTCT	2700
GCCGTGGCCA	GGACAACGTA	TACTCATCAG	ATAACAGCAA	TACCTGATCA	2750
CTACTTCGCA	CTAGTTCTC	GGTACTATGC	ATATGATCCA	ATATCAAAGG	2800
AAATGATAGC	ATTGAAGGAT	GAGACTAAC	CAATTGAGGA	GTGGCAGCAT	2850
ATAGAACAGC	TAAAGGGTAG	TGCTGAAGGA	AGCATAACGAT	ACCCCGCATG	2900
GAATGGGATA	ATATCACAGG	AGGTACTAGA	CTACCTTCA	TCCTACATAA	2950
ATAGACGCAT	ATAAGTACGC	ATTAAAGCAT	AAACACGCAC	TATGCCGTT	3000
TTCTCATGTA	TATATATATA	CAGGCAACAC	GCAGATATAG	GTGCGACGTG	3050
AAACAGTGAGC	TGTATGTGCG	CAGCTCGCGT	TGCATTTTCG	GAAGCGCTCG	3100
TTTCGGAAA	CGCTTGAAG	TTCCTATTCC	GAAGTTCCTA	TTCTCTAGAA	3150
AGTATAGGAA	CTTCAGAGCG	CTTTGAAAAA	CCAAAAGCGC	TCTGAAGACG	3200
CACTTCAAA	AAACCAAAAA	CGCACCGGAC	TGTAACGAGC	TACTAAAATA	3250
TTGCGAATAC	CGCTTCCACA	AACATTGCTC	AAAAGTATCT	CTTTGCTATA	3300
TATCTCTGTG	CTATATCCCT	ATATAACCTA	CCCATCCACC	TTTCGCTCCT	3350
TGAACCTGCA	TCTAAACTCG	ACCTCTACAT	TTTTTATGTT	TATCTCTAGT	3400
ATTACTCTT	AGACAAAAAA	ATTGTAGTAA	GAACATTCTA	TAGAGTGAAT	3450
CGAAAACAAT	ACGAAAATGT	AAACATTTC	TATACGTAGT	ATATAGAGAC	3500
AAAATAGAAG	AAACCGTTCA	TAATTTC	ACCAATGAAG	AATCATCAAC	3550
GCTATCACTT	TCTGTTCACA	AAGTATGCGC	AATCCACATC	GGTATAGAAT	3600
ATAATCGGGG	ATGCC	CTTGA	TGCACCCGCA	GCTTCGCTAG	3650
TAATCAGTAA	ACGCGGGAAG	TGGAGTCAGG	CTTTTTTAT	GGAGAGAAA	3700
ATAGACACCA	AACTGACCTT	CTTCTAACCT	TAACGGACCT	ACAGTGC	3750
AAGTTATCAA	GAGACTGCAT	TATAGAGCGC	ACAAAGGAGA	AAAAAAGTAA	3800
TCTAAGATGC	TTTGTAGAA	AAATAGCGCT	CTCGGGATGC	ATTTTGTAG	3850
AACAAAAAAG	AACTGATAGAT	TCTTTGTTGG	AAAAATAGCG	CTCTCGCGTT	3900
GCATTTCTGT	TCTGAAAAA	TGCAGCTCAG	ATTCTTGT	TGAAAATTAA	3950
GCGCTCTCGC	GTTGCATT	TGTTTACAA	AAATGAAGCA	CAGATTCTC	4000
GTTGGTAAAA	TAGCGCTT	GCCTTGCATT	TCTGTTCTGT	AAAAATGCAG	4050
CTCAGATTCT	TTGTTGAAA	AATTAGCGCT	CTCGCGTTGC	ATTTTGTTC	4100
TACAAAATGA	AGCACAGATG	CTTCGTTAAC	AAAGATATGC	TATTGAAGTG	4150
CAAGATGGAA	ACGCAGAAAA	TGAACCGGGG	ATGCGACGTG	CAAGATTACC	4200
TATGCAATAG	ATGCAATAGT	TTCTCCAGGA	ACCGAAATAC	ATACATTGTC	4250
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CTGTTTCAGG	GAAAACCTCCC	AGGTTCGGAT	GTTCAAAATT	CAATGATGGG	4350
TAACAAGTAC	GATCGTAAAT	CTGTAAAACA	GTTTGTCCGA	TATTAGGCTG	4400

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TATCTCCTCA	AAGCGTATTG	GAATATCATT	GAGAAGCTGC	ATTTTTTTTT	4450
TTTTTTATAT	ATATTTCAAG	GATATACCAT	TGTAATGCCT	GCCCCTAAGA	4500
AGATCGTCGT	TTGCCAGGT	GACCACGTTG	GTCAAGAAAT	CACAGCCGAA	4550
GCCATTAAGG	TTCTTAAAGC	TATTTCTGAT	GTTCGTTCCA	ATGTCAAGTT	4600
CGATTTGAA	AATCATTAA	TTGGTGGTGC	TGCTATCGAT	GCTACAGGTG	4650
TTCCACTTCC	AGATGAGGCG	CTGGAAGCCT	CCAAGAAGGC	TGATGCCGTT	4700
TTGTTAGGTG	CTGTGGGTGG	TCCTAAATGG	GGTACCGGT	GTGTTAGACC	4750
TGAACAAGGT	TTACTAAAAA	TCCGTAAAGA	ACTTCAATTG	TACGCCAACT	4800
TAAGACCATG	TAACTTGCA	TCCGACTCTC	TTTTAGACTT	ATCTCCAATC	4850
AAGCCACAAT	TTGCTAAAGG	TACTGACTTC	GTTGTTGTTA	GAGAATTAGT	4900
GGGAGGTATT	TACTTTGGTA	AGAGAAAGGA	AGACGATGGT	GATGGTGTG	4950
CTTGGGATAG	TGAACAATAC	ACCGTTCCAG	AAGTGCAAAG	AATCACAAAGA	5000
ATGGCCGCTT	TCATGGCCCT	ACAACATGAG	CCACCATTGC	CTATTTGGTC	5050
CTTGGATAAA	GCTAATGTT	TGGCCTCTTC	AAGATTATGG	AGAAAAACTG	5100
TGGAGGAAAC	CATCAAGAAC	GAATTCCCTA	CATTGAAAGT	TCAACATCAA	5150
TTGATTGATT	CTGCCGCCAT	GATCCTAGTT	AAGAACCCAA	CCCACCTAAA	5200
TGGTATTATA	ATCACCCAGCA	ACATGTTGG	TGATATCATC	TCCGATGAAG	5250
CCTCCGTTAT	CCCAGGCTCC	TTGGGTTTGT	TGCCATCTGC	GTCCTTGGCC	5300
TCTTGCCAG	ACAAGAACAC	CGCATTGGT	TTGTACGAAC	CATGCCATGG	5350
TTCCGCTCCA	GATTGCCAA	AGAATAAGGT	CAACCCTATC	GCCACTATCT	5400
TGTCTGCTGC	AATGATGTTG	AAATTGTCAT	TGAACTTGCC	TGAAGAAGGT	5450
AAAGCCATTG	AAGATGCAGT	AAAAAAAGTT	TTGGATGCAG	GTATCAGAAC	5500
TGGTGATTTA	GGTGGTTCCA	ACAGTACCAAC	CGAAGTCGGT	GATGCTGTG	5550
CCGAAGAAGT	TAAGAAAATC	CTTGCTTAAA	AAGATTCTCT	TTTTTTATGA	5600
TATTGTCACA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	5650
AAAAAAAAAA	AAAATGCAGC	GTCACATCGG	ATAATAATGA	TGGCAGCCAT	5700
TGTAGAAGTG	CCTTTGCAT	TTCTAGTCTC	TTTCTCGGTC	TAGCTAGTTT	5750
TACTACATCCG	CGAAGATAGA	ATCTTAGATC	ACACTGCCTT	TGCTGAGCTG	5800
GATCAATAGA	GTAACAAAAG	AGTGGTAAGG	CCTCGTTAAA	GGACAAGGAC	5850
CTGAGCGGAA	GTGTATCGTA	CAGTAGACGG	AGTATACTAG	TATAGTCTAT	5900
AGTCCGTGGA	ATTCTCATGT	TTGACAGCTT	ATCATCGATA	AGCTAGCTTT	5950
CTAACTGATC	TATCCAAAAC	TGAAAATTAC	ATTCTTGATT	AGGTTTATCA	6000
CAGGCAAATG	TAATTGTGG	TATTTGCCG	TTCAAAATCT	GTAGAATT	6050
CTCATTGGTC	ACATTACAAC	CTGAAAATAC	TTTATCTACA	ATCATACCAT	6100
TCTTAATAAC	ATGCCCCCTT	AATACTAGGA	TCAGGCATGA	ACGCATCACA	6150
GACAAAATCT	TCTTGACAAA	CGTCACAATT	GATCCCTCCC	CATCCGTTAT	6200
CACAATGACA	GGTGTCAATT	TGTGCTCTTA	TGGGACGATC	CTTATTACCG	6250
CTTTCATCCG	GTGATTGACC	GCCACAGAGG	GGCAGAGAGC	AATCATCACC	6300
TGCAAACCCCT	TCTATACACT	CACATCTACC	AGTGATCGAA	TTGCATTCA	6350
AAAACGTGTT	GCATTCAAAA	ATAGGTAGCA	TACAATTAAA	ACATGGCGGG	6400
CATGTATCAT	TGCCCTTATC	TTGTGCAAGT	AGACCGAAT	TTTTCGAAGA	6450
AGTACCTTCA	AAGAATGGGG	TCTTATCTTG	TTTGCAGT	ACCACTGAGC	6500
AGGATAATAA	TAGAAATGAT	AATATACTAT	AGTAGAGATA	ACGTGCGATGA	6550
CTTCCCATAC	TGTAATTGCT	TTTAGTTGTG	TATTTTTAGT	GTGCAAGTTT	6600
CTGTAATCG	ATTAATT	TTTCTTTCC	TCTTTTATT	AACCTTAATT	6650
TTTATTTAG	ATTCTGACT	TCAACTCAAG	ACGCACAGAT	ATTATAACAT	6700
CTGCATAATA	GGCATTGCA	AGAATTACTC	GTGAGTAAGG	AAAGAGTGAG	6750
GAACATATCGC	ATACCTGCAT	TTAAAGATGC	CGATTGGC	GCGAATCCTT	6800
TATTTTGGCT	TCACCCCTCAT	ACTATTATCA	GGGCCAGAAA	AAGGAAGTGT	6850

TTCCCTCCTT	CTTGAATTGA	TGTTACCCCTC	ATAAAGCACG	TGGCCTCTTA	6900
TCGAGAAAGA	AATTACCGTC	GCTCGTGATT	TGTTTGCAAA	AAGAACAAAA	6950
CTGAAAAAAC	CCAGACACGC	TCGACTTCCT	GTCTTCCTAT	TGATTGCAGC	7000
TTCCAATTTC	GTCACACAAAC	AAGGTCCCTAG	CGACGGCTCA	CAGGTTTTGT	7050
AACAAGCAAT	CGAAGGTTCT	GGAAATGGCGG	GGAAAGGGTT	TAGTACCAACA	7100
TGCTATGATG	CCCACGTGA	TCTCCAGAGC	AAAGTTCGTT	CGATCGTACT	7150
GTACTCTCTC	TCTTTCAAAC	AGAATTGTCC	GAATCGTGTG	ACAACAAACAG	7200
CCTGTTCTCA	CACACTCTTT	TCTTCTAACCC	AAGGGGGTGG	TTTAGTTTAG	7250
TAGAACCTCG	TGAAACTTAC	ATTTACATAT	ATATAAACTT	GCATAAAATTG	7300
GTCAATGGAA	GAAATACATA	TTTGGTCTTT	TCTAATTCTGT	AGTTTTCAA	7350
GTTCTTAGAT	GCTTCTTTT	TCTCTTTTTT	ACAGATCATC	AAGGAAGTAA	7400
TTATCTACTT	TTTACAACAA	ATACAAAAGA	TCTATGAGAT	TTCCCTCAAT	7450
TTTTACTGCA	GTTTATTTCG	CAGCATCCTC	CGCATTAGCT	GCTCCAGTCA	7500
ACACTACAAC	AGAAGATGAA	ACGGCACAAA	TTCCGGCTGA	AGCTGTCATC	7550
GGTTACTTAG	ATTTAGAAGG	GGATTTCGAT	GTTGCTGTTT	TGCCATTTC	7600
CAACAGCACA	AATAACGGGT	TATTGTTTAT	AAATACTACT	ATTGCCAGCA	7650
TTGCTGCTAA	AGAAGAAGGG	GTAAGCTTGG	ATAAAAGAAA	CAGCGACTCT	7700
GAATGCCCGC	TGAGCCATGA	TGGCTACTGC	CTGCACGACG	GTGTATGCAT	7750
GTATATCGAA	GCTCTGGACA	AATACGCATG	CAACTGCGTA	GTTGGTTACA	7800
TCGGCGAACG	TTGCCAGTAC	CGCGACCTGA	AATGGTGGGA	GCTCCGTTAA	7850
TAAGGATCC					7859

\*\*\*\*\* END OF SEQ ID NO: 4 \*\*\*\*\*

75

SEQ. ID NO:5

SEQUENCE TYPE: nucleotide

SEQUENCE LENGTH: 15 base pairs

FEATURES: Top strand of adapter to fuse C-terminal  
end of the  $\alpha$ -factor pro-peptide to  
synthetic hirudin gene

SEQUENCE:

AGCTTGGATA AAAGA

15

\*\*\*\*\* END OF SEQ ID NO: 5 \*\*\*\*\*

**SUBSTITUTE SHEET**

SEQ. ID NO:6

SEQUENCE TYPE: nucleotide

SEQUENCE LENGTH: 11 base pairs

FEATURES:

Bottom strand of adapter to fuse C-terminal end of the  $\alpha$ -factor pro-peptide to synthetic hirudin gene

SEQUENCE:

TCTTTTATCC A

11

\*\*\*\*\* END OF SEQ ID NO: 6 \*\*\*\*\*

**SUBSTITUTE SHEET**

SEQ ID NO:7

SEQUENCE TYPE: nucleotide  
SEQUENCE LENGTH: 223 base pairs  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SOURCE: synthetic  
FEATURES: hirudin type HV-1 gene with 5 amino acid adaptor (corresponding to C-terminus of alpha factor) at amino terminus.  
from 1 to 6 bp (AAGCTT) is HindIII site  
from 118 to 123 bp (GGATCC) is BamHI site.

SEQUENCE:

AAGCTTGGAT	AAAAGAGTTG	TTTACACCGA	CTGTACTGAA	TCCGGACAAA	50
ACCTGTGTTT	GTGTGAGGGT	TCTAACGTCT	GTGGTCAGGG	TAACAAATGC	100
ATCCTGGGTT	CCGACGGTGA	AAAGAACCAA	TGTGTCACTG	GTGAAGGTAC	150
CCCAAAGCCG	CAGTCCCACA	ACGATGGAGA	TTTCGAAGAA	ATCCCAGAAG	200
AATATCTGCA	GTAATAGGGA	TCC			223

\*\*\*\*\* END OF SEQ ID NO: 7 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ ID NO:8

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 420 base pairs  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 FEATURES: Factor Xa-cleavable Hirudin-IEGR-Hirudin  
 SEQUENCE:

GTT GTT TAC ACC GAC TGT ACT GAA TCC GGA CAA AAC CTG TGT	42
Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys	
5	10
TTG TGT GAG GGT TCT AAC GTC TGT GGT CAG GGT AAC AAA TGC	84
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys	
15	20
ATC CTG GGT TCC GAC GGT GAA AAG AAC CAA TGT GTC ACT GGT	126
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly	
30	35
GAA GGT ACC CCA AAG CCG CAG TCC CAC AAC GAT GGA GAT TTC	168
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe	
45	50
GAA GAA ATC CCA GAA GAA TAT CTG CAG ATC GAA GGA AGA GTT	210
Glu Glu Ile Pro Glu Glu Tyr Leu Gln Ile Glu Gly Arg Val	
60	65
GTT TAC ACC GAC TGT ACT GAA TCC GGA CAA AAC CTG TGT TTG	252
Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys Leu	
75	80
TGT GAG GGT TCT AAC GTC TGT GGT CAG GGT AAC AAA TGC ATC	294
Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys Ile	
85	90
CTG GGT TCC GAC GGT GAA AAG AAC CAA TGT GTC ACT GGT GAA	336
Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly Glu	
100	105
GGT ACC CCA AAG CCG CAG TCC CAC AAC GAT GGA GAT TTC GAA	378
Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe Glu	
115	120
GAA ATC CCA GAA GAA TAT CTG CAG TAATAGGGAT CCGAATTTC	420
Glu Ile Pro Glu Glu Tyr Leu Gln	
130	

\*\*\*\*\* END OF SEQ ID NO: 8 \*\*\*\*\*

SEQ. ID NO:13

SEQUENCE TYPE: nucleotide  
SEQUENCE LENGTH: 17 base pairs  
FEATURES: Primers for dideoxy sequencing of  
streptokinase gene

SEQUENCE:

5'-CACTATCAGTAGCAAAT-3'	BB 3510
5'-TGGTCTAACGCGCACAT-3'	BB 2136
5'-GAGTAAACTGTACAGTA-3'	BB 3509
5'-GATCTCATAAGCTTGTT-3'	BB 3508
5'-TTTAGCCTTATCACGAG-3'	BB 2135
5'-GACACCAACCGTATCAT-3'	BB 2753
5'-CGTTGATGTCAACACCA-3'	BB 3718
5'-GCTATCGGTGACACCAT-3'	BB 2754
5'-GACGACTACTTGAGGT-3'	BB 2755
5'-CCCAACCTGTCCAAGAA-3'	BB 2134

\*\*\*\*\* END OF SEQ ID NO: 13 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ. ID NO:14

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1335 base pairs  
 FEATURES: Streptokinase gene from S. equisimilis  
 SEQUENCE:

GAATTCATGAAAAATTACTTATCTTTGGGATGTTGCACTGCTGTTGCACTAACATTT  
 MetLysAsnTyrLeuSerPheGlyMetPheAlaLeuLeuPheAlaLeuThrPhe  
 GGAACAGTCAATTCTGTCCAAGCTATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCT  
 GlyThrValAsnSerValGlnAlaIleAlaGlyProGluTrpLeuLeuAspArgProSer  
 GTCAACAAACAGCCAATTAGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGAC  
 ValAsnAsnSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAsp  
 ATTAGTCTTAAATTGGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACA  
 IleSerLeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLysThr  
 GAGCAAGGCTTAAGTCCAAAATCAAACCATTTGCTACTGATAGTGGCGCGATGCCACAT  
 GluGlnGlyLeuSerProLysSerProPheAlaThrAspSerGlyAlaMetProHis  
 AAACTTGAAAAAGCTGACTTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCAC  
 LysLeuGluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHis  
 AGTAACGACGACTACTTGAGGTCAATTGATTTGCAAGCGATGCAACCATTACTGATCGA  
 SerAsnAspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArg  
 AACGGCAAGGTCTACTTGCTGACAAAGATGGTCGGTAACCTGGCGACCCAACCTGTC  
 AsnGlyLysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProVal  
 CAAGAATTTTGCTAACGGACATGTGCGCGTTAGACCATAAAAGAAAAACCAATACAA  
 GlnGluPheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGln  
 AATCAAGCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCCCTGAT  
 AsnGlnAlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAsp  
 GACGATTTCAGACCAGGTCTCAAAGATACTAACGCTATTGAAAACACTAGCTATCGGTGAC  
 AspAspPheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAsp  
 ACCATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTAAACAAACCCATCCA  
 ThrIleThrSerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisPro  
 GGCTATACGATTTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTCCGT  
 GlyTyrThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArg  
 ACGATTACCAATGGATCAAGAGTTACTTACCATGTCAAAATCGGGACAAGCTTAT  
 ThrIleLeuProMetAspGlnGluPheThrHisValLysAsnArgGluGlnAlaTyr

SUBSTITUTE SHEET

GAGATCAATAAAAATCTGGTCTGAATGAAGAAATAACAAACACTGACCTGATCTCTGAG  
GluIleAsnLysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGlu  
AAATATTACGTCTTAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCACTTG  
LysTyrTyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeu  
AAACTGTTCACCATCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAAGCGAGCAG  
LysLeuPheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGluGln  
CTCTTAACAGCTAGCGAACGTAACCTAGACTTCAGAGATTATACGATCCTCGTGATAAG  
LeuLeuThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLys  
GCTAAACTACTCTACAACAATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAA  
AlaLysLeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGly  
AAAGTAGAAGATAATCACGATGACACCAACCGTATCATAACCGTTATATGGGCAAGCGA  
LysValGluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArg  
CCCGAAGGAGAGAATGCTAGCTATCATTAGCCTATGATAAAAGATCGTTACCGAAGGAA  
ProGluGlyGluAsnAlaSerTyrHisLeuAlaTyrAspLysAspArgTyrThrGluGlu  
GAACGAGAAGTTACAGCTACCTGCGTTATACAGGGACACCTATACTGATAACCCTAAC  
GluArgGluValTyrSerTyrLeuArgTyrThrGlyThrProIleProAspAsnProAsn  
GACAAATAAGGATCC\*  
AspLysEnd

\*\*\*\*\* END OF SEQ ID NO: 14 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ. ID NO:17

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1317 base pairs  
 FEATURES: OmpAL fused to mature streptokinase gene  
 SEQUENCE:

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CATATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTCGCGACCGTAGCG
M K K T A I A I A V A L A G F A T V A

CAGGCCATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAACAAACAGCCAATTA
Q A I A G P E W L L D R P S V N N S Q L

GTTGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATTTTT
V V S V A G T V E G T N Q D I S L K F F

GAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCA
E I D L T S R P A H G G K T E Q G L S P

AAATCAAAACCATTGCTACTGATAGTGGCGCATGCCACATAACTTGAAAAAGCTGAC
K S K P F A T D S G A M P H K L E K A D

TTACTAAAGGCTATTCAAGAACAAATTGATCGCTAACGTCCACAGTAACGACGACTACTTT
L L K A I Q E Q L I A N V H S N D D Y F

GAGGTCAATTGATTTGCAAGCGATGCAACCATTACTGATCGAAACGGCAAGGTCTACTTT
E V I D F A S D A T I T D R N G K V Y F

GCTGACAAAGATGGTCGGTAACCTGCCAACCTGTCCAAGAATTTTGCTAAGC
A D K D G S V T L P T Q P V Q E F L L S

GGACATGTGCCCGTTAGACCATATAAGAAAAACCAATACAAATCAAGCGAAATCTGTT
G H V R V R P Y K E K P I Q N Q A K S V

GATGTGGAATATACTGTACAGTTACTCCCTTAAACCCCTGATGACGATTCAGACCGAGT
D V E Y T V Q F T P L N P D D D F R P G

CTCAAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACCATCACATCTCAAGAA
L K D T K L L K T L A I G D T I T S Q E

TTACTAGCTCAAGCACAAAGCATTAAACAAACCCATCCAGGCTATACGATTTATGAA
L L A Q A Q S I L N K T H P G Y T I Y E

CGTGACTCCTCAATCGCACTCATGACAATGACATTTCGGTACGATTTACCAATGGAT
R D S S I V T H D N D I F R T I L P M D

CAAGAGTTTACTTACCATGTAAAAATCGGGAACAAAGCTTATGAGATCAATAAAAATCT
Q E F T Y H V K N R E Q A Y E I N K K S

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SUBSTITUTE SHEET

GGTCTGAATGAAGAAATAAACAAACTGACCTGATCTCTGAGAAATATTACGTCTTAAA  
G L N E E I N N T D L I S E K Y Y V L K  
AAAGGGAAAAGCGTATGATCCCTTGATCGCAGTCACTTGAAACTGTTACCATCAAA  
K G E K P Y D P F D R S H L K L F T I K  
TACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTCTAACAGCTAGCGAA  
Y V D V N T N E L L K S E Q L L T A S E  
CGTAACCTAGACTTCAGAGATTATACGATCCTCGTGATAAGGCTAAACTACTCTACAAC  
R N L D F R D L Y D P R D K A K L L Y N  
AATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAAGTAGAAGATAATCAC  
N L D A F G I M D Y T L T G K V E D N H  
GATGACACCAACCGTATCATAACCGTTATATGGCAAGCGACCCGAAGGAGAGAATGCT  
D D T N R I I T V Y M G K R P E G E N A  
AGCTATCATTAGCCTATGATAAAGATCGTTATACCGAAGAAGAACGAGAAAGTTACAGC  
S Y H L A Y D K D R Y T E E E R E V Y S  
TACCTGCCTTATACAGGGACACCTATACCTGATAACCCTAACGACAAATAAGGATCC\*  
Y L R Y T G T P I P D N P N D K \*  
\*\*\*\*\* END OF SEQ ID NO: 17 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ. ID NO:23

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1197 nucleotides  
 FEATURES: Methionyl-streptokinase fusion protein  
 SEQUENCE:

CATATGATTGCTGGACCTGAGTGGCTGCTAGACCGTCATCTGTCAACAAACAGCCAATTA  
 MetIleAlaGlyProGluTrpLeuLeuAspArgProSerValAsnAsnSerGlnLeu  
 GTTGTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATTTTT  
 ValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIleSerLeuLysPhePhe  
 GAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCA  
 GluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGluGlnGlyLeuSerPro  
 AAATCAAACCATTGCTACTGATAGTGGCGCGATGCCACATAAACTTGAAAAAGCTGAC  
 LysSerLysProPheAlaThrAspSerGlyAlaMetProHisLysLeuGluLysAlaAsp  
 TTACTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCACAGTAACGACGACTACTTT  
 LeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSerAsnAspAspTyrPhe  
 GAGGT CATTGATTTGCAAGCGATGCAACCATTACTGATCGAAACGGCAAGGTCTACTTT  
 GluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsnGlyLysValTyrPhe  
 GCTGACAAAGATGGTCGTAACCTGCCGACCCAACCTGTCCAAGAATTGGCTAACGC  
 AlaAspLysAspGlySerValThrLeuProThrGlnProValGlnGluPheLeuLeuSer  
 GGACATGTGCGCGTTAGACCATAAAAGAAAAACCAATAACAAATCAAGCGAAATCTGTT  
 GlyHisValArgValArgProTyrLysGluLysProIleGlnAsnGlnAlaLysSerVal  
 GATGTGGAATATACTGTACAGTTACTCCCTAAACCCCTGATGACGATTTCAGACCAGGT  
 AspValGluTyrThrValGlnPheThrProLeuAsnProAspAspAspPheArgProGly  
 CTCAAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACCACATCTCAAGAA  
 LeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThrIleThrSerGlnGlu  
 TTACTAGCTCAAGCACAAAGCATTAAACAAACCCATCCAGGCTATACGATTATGAA  
 LeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGlyTyrThrIleTyrGlu  
 CGTGA CTCCTCAATCGTCACTCATGACAATGACATTCCCGTACGATTTCACCAATGGAT  
 ArgAspSerSerIleValThrHisAspAsnAspIlePheArgThrIleLeuProMetAsp  
 CAAGAGTTTACTTACCATGTAAAAATCGGGAACAAAGCTTATGAGATCAATAAAAATCT  
 GlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGluIleAsnLysSer  
 GGTCTGAATGAAGAAATAAACACACTGACCTGATCTCTGAGAAATTACGTCTTAAA  
 GlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLysTyrTyrValLeuLys

SUBSTITUTE SHEET

AAAGGGAAAAGCCGTATGCCCTTGATCGCAGTCACTTGAAACTGTTCACCATCAA  
LysGlyGluLysProTyrAspProPheAspArgSerHisLeuLysLeuPheThrIleLys  
TACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTCTAACAGCTAGCGAA  
TyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeuLeuThrAlaSerGlu  
CGTAACCTAGACTTCAGAGATTTACGATCCTCGTGATAAGGCTAAACTACTCTACAAC  
ArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAlaLysLeuLeuTyrAsn  
AATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAGTAGAAGATAATCAC  
AsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLysValGluAspAsnHis  
GATGACACCAACCGTATCATAACCGTTATATGGCAAGCGACCCGAAGGAGAGAATGCT  
AspAspThrAsnArgIleIleThrValTyrMetGlyLysArgProGluGlyGluAsnAla  
AGCTATCATTAGCCTATGATAAAGATCGTTATACCGAAGAAGAACGAGAAGTTACAGC  
SerTyrHisLeuAlaTyrAspLysAspArgTyrThrGluGluGluArgGluValTyrSer  
TACCTGCCTTATACAGGGACACCTATACCTGATAACCCTAACGACAAATAAGGATCC\*  
TyrLeuArgTyrThrGlyThrProIleProAspAsnProAsnAspLysEnd

\*\*\*\*\* END OF SEQ ID NO: 23 \*\*\*\*\*

**SUBSTITUTE SHEET**

SEQ. ID NO:24

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1513 nucleotides  
 FEATURES: Streptokinase fused to yeast  $\alpha$ -factor  
 SEQUENCE:

AGATCTATGAGATTCCCTCAATTTTACTGCAGTTTATTGCCAGCATCCTCCGCATTA  
 MetArgPheProSerIlePheThrAlaValLeuPheAlaAlaSerSerAlaLeu  
 GCTGCTCCAGTCACACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTC  
 AlaAlaProValAsnThrThrGluAspGluThrAlaGlnIleProAlaGluAlaVal  
 ATCGGTTACTTAGATTAGAAGGGGATTTCGATGTTGCTGTTGCCATTTCACAGC  
 IleGlyTyrLeuAspLeuGluGlyAspPheAspValAlaValLeuProPheSerAsnSer  
 ACAAAATAACGGGTATTGTTATAAAACTACTATTGCCAGCATTGCTGCTAAAGAACAGAA  
 ThrAsnAsnGlyLeuLeuPheIleAsnThrThrIleAlaSerIleAlaAlaLysGluGlu  
 GGGGTAAGCTTGGATAAAAGAATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTC  
 GlyValSerLeuAspLysArgIleAlaGlyProGluTrpLeuLeuAspArgProSerVal  
 AACAAACAGCCAATTAGTTGTTAGCGTGCTGGTACTGTTGAGGGGACGAATCAAGACATT  
 AsnAsnSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIle  
 AGTCTTAAATTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAG  
 SerLeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGlu  
 CAAGGCTTAAGTCCAAAATCAAACCATTTGCTACTGATAGTGGCGCGATGCCACATAAA  
 GlnGlyLeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetProHisLys  
 CTTGAAAAAGCTGACTTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCACAGT  
 LeuGluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSer  
 AACGACGACTACTTGAGGTATTGATTGCAAGCGATGCAACCATTACTGATCGAAAC  
 AsnAspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsn  
 GGCAAGGTCTACTTGCTGACAAAGATGGTTGGTAACCTTGCCTGACCCAACTGTCCAA  
 GlyLysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProValGln  
 GAATTTTGCTAAGCGGACATGTGCGCGTTAGACCATATAAGAAAAACCAATACAAAT  
 GluPheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGlnAsn  
 CAAGCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCTGATGAC  
 GlnAlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAspAsp  
 GATTTCAGACCAGGTCTCAAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACC  
 AspPheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThr

ATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTAAACAAAACCCATCCAGGC  
IleThrSerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGly  
  
TATACGATTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTCCGTACG  
TyrThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArgThr  
  
ATTTTACCAATGGATCAAGAGTTACTTACCATGTCAAAAATCGGAACAAGCTTATGAG  
IleLeuProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGlu  
  
ATCAATAAAAATCTGGTCTGAATGAAGAAATAAACAACTGACCTGATCTCTGAGAAA  
IleAsnLysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLys  
  
TATTACGTCCCTAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCAC TGAGAAA  
TyrTyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeuLys  
  
CTGTTACCACCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTC  
LeuPheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeu  
  
TTAACAGCTAGCGAACGTAACCTAGACTTCAGAGATTATACGATCCTCGTGATAAGGCT  
LeuThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAla  
  
AAACTACTCTACAACAATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAAA  
LysLeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLys  
  
GTAGAAAGATAATCACGATGACACCAACCGTATCATAACC GTTTATATGGCAAGCGACCC  
ValGluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArgPro  
  
GAAGGGAGAGAATGCTAGCTATCATTAGCCTATGATAAAAGATCGTTATACCGAAGAAGAA  
GluGlyGluAsnAlaSerTyrHisLeuAlaTyrAspLysAspArgTyrThrGluGluGlu  
  
CGAGAAGTTACAGCTACCTGCGTTATACAGGGACACCTATACCTGATAACCCTAACGAC  
ArgGluValTyrSerTyrLeuArgTyrThrGlyThrProIleProAspAsnProAsnAsp  
  
AAATAAGGATCC\*  
LysEnd

\*\*\*\*\* END OF SEQ ID NO: 24 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ. ID NO:26

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1120 nucleotides  
 FEATURES: Truncated Met-streptokinase (aa 16-383)  
 SEQUENCE:

CATATGAGCCAATTAGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATT  
 MetSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIle  
 AGTCTTAAATTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAG  
 SerLeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGlu  
 CAAGGCTTAAGTCCAAAATCAAACCATTGCTACTGATAGTGGCGCGATGCCACATAAA  
 GlnGlyLeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetProHisLys  
 CTTGAAAAAGCTGACTTACTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCACAGT  
 LeuGluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSer  
 AACGACGACTACTTGAGGTCATTGATTTGCAAGCGATGCAACCATTACTGATCGAAAC  
 AsnAspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsn  
 GGCAAGGTCTACTTGCTGACAAAGATGGTCGGTAACCTGCCGACCCACCTGTCCAA  
 GlyLysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProValGln  
 GAATTTGCTAAGCGGACATGTGCGCGTTAGACCATATAAGAAAACCAATACAAAAT  
 GluPheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGlnAsn  
 CAAGCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCCCTGATGAC  
 GlnAlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAspAsp  
 GATTCAGACCAGGTCTCAAAGATACTAACGCTATTGAAAACACTAGCTATCGGTGACACC  
 AspPheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThr  
 ATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTAAACAAAACCCATCCAGGC  
 IleThrSerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGly  
 TATACGATTATGAAACGTGACTCCTCAATCGTCACTCATGACAATGACATTCCCGTACG  
 TyrThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArgThr  
 ATTTTACCAATGGATCAAGAGTTACTTACCATGTCAAAAATCGGGAACAAAGCTTATGAG  
 IleLeuProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGlu  
 ATCAATAAAAATCTGGTCTGAATGAAGAAATAACAAACACTGACCTGATCTCTGAGAAA  
 IleAsnLysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLys  
 TATTACGTCTAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCACGGAAA  
 TyrTyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeuLys

SUBSTITUTE SHEET

CTGTTCACCATCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAAGCGAGCAGCTC  
LeuPheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGlnLeu  
TTAACAGCTAGCGAACGTAACCTAGACTTCAGAGATTATACGATCCTCGTGATAAGGCT  
LeuThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAla  
AAACTACTCTACAACAATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAAA  
LysLeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLys  
GTAGAAGATAATCACGATGACACCAACCGTATCATAACCCTTATATGGGCAAGCGACCC  
ValGluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArgPro  
GAAGGGAGAGAATGCTAGCTATCATTAGCCTAAGGATCC\*  
GluGlyGluAsnAlaSerTyrHisLeuAlaEnd

\*\*\*\*\* END OF SEQ ID NO: 26 \*\*\*\*\*

SUBSTITUTE SHEET

SEQ. ID NO:29

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 2590 nucleotides  
 FEATURES: OmpAL-Streptokinase-streptokinase fusion  
 linked by thrombin-cleavable VELQGVVPRG  
 SEQUENCE:

CATATGAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTCGCGACCGTAGCG  
 MetLysLysThrAlaIleAlaIleAlaValAlaLeuAlaGlyPheAlaThrValAla  
 CAGGCCATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAACAAACAGCCAATT  
 GlnAlaIleAlaGlyProGluTrpLeuLeuAspArgProSerValAsnAsnSerGlnLeu  
 GTTGTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATT  
 ValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIleSerLeuLysPhePhe  
 GAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCA  
 GluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGluGlnGlyLeuSerPro  
 AAATCAAAACCATTGCTACTGATAGTGGCGCGATGCCACATAAAACTTGAAAAAGCTGAC  
 LysSerLysProPheAlaThrAspSerGlyAlaMetProHisLysLeuGluLysAlaAsp  
 TTACTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCACAGTAACGACGACTACTTT  
 LeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSerAsnAspAspTyrPhe  
 GAGGTCAATTGATTTGCAAGCGATGCAACCATTACTGATCGAAACGGCAAGGTCTACTTT  
 GluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsnGlyLysValTyrPhe  
 GCTGACAAAGATGGTTGGTAACCTGGCAGCCAACCTGTCCAAGAATTGGCTAAC  
 AlaAspLysAspGlySerValThrLeuProThrGlnProValGlnGluPheLeuLeuSer  
 GGACATGTGCGCGTTAGACCATAAAAGAAAAACCAATAACAAATCAAGCGAAATCTGTT  
 GlyHisValArgValArgProTyrLysGluLysProIleGlnAsnGlnAlaLysSerVal  
 GATGTGGAATATACTGTACAGTTACTCCCTAAACCCCTGATGACGATTTCAGACCAGGT  
 AspValGluTyrThrValGlnPheThrProLeuAsnProAspAspPheArgProGly  
 CTCAAAGATACTAACGCTATTGAAAACACTAGCTATCGGTGACACCACATCTCAAGAA  
 LeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThrIleThrSerGlnGlu  
 TTACTAGCTCAAGCACAAAGCATTTAACAAAACCCATCCAGGCTATACGATTTATGAA  
 LeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGlyTyrThrIleTyrGlu  
 CGTGACTCCTCAATCGTCACTCATGACAATGACATTTCGGTACGATTTCACCAATGGAT  
 ArgAspSerSerIleValThrHisAspAsnAspIlePheArgThrIleLeuProMetAsp  
 CAAGAGTTTACTTACCATGTAAAAATCGGAAACAAGCTTATGAGATCAATAAAATCT  
 GlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGluIleAsnLysSer

SUBSTITUTE SHEET

GGTCTGAATGAAGAAATAAACAAACACTGACCTGATCTCTGAGAAATATTACGTCTTAAA  
 GlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLysTyrTyrValLeuLys  
 AAAGGGGAAAAGCCGTATGCCCTTGATCGCAGTCACCTGAAACTGTTCACCATCAAA  
 LysGlyGluLysProTyrAspProPheAspArgSerHisLeuLysLeuPheThrIleLys  
 TACGTTGATGTCAACACCAACGAATTGCTAAAAAGCGAGCAGCTCTAACAGCTAGCGAA  
 TyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeuLeuThrAlaSerGlu  
 CGTAACCTAGCTCAGAGATTATACGATCCTCGTGATAAGGCTAAACTACTCTACAAC  
 ArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAlaLysLeuLeuTyrAsn  
 AATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAGTAGAAGATAATCAC  
 AsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLysValGluAspAsnHis  
 GATGACACCAACCGTATCATAACCCTTATATGGCAAGCGACCCGAAGGAGAGAATGCT  
 AspAspThrAsnArgIleIleThrValTyrMetGlyLysArgProGluGlyGluAsnAla  
 AGCTATCATTAGCCTATGATAAAGATCGTTACCGAAGAACGAGAGAAGTTACAGC  
 SerTyrHisLeuAlaTyrAspLysAspArgTyrThrGluGluArgGluValTyrSer  
 TACCTGCCTTACAGGGACACCTACCTGATAACCTAACGACAAAGTAGAGCTGCAG  
 TyrLeuArgTyrThrGlyThrProIleProAspAsnProAsnAspLysValGluLeuGln  
 GGAGTAGTTCTCGTGGATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAAC  
 GlyValValProArgGlyIleAlaGlyProGluTrpLeuLeuAspArgProSerValAsn  
 AACAGCCAATTAGTTGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATTAGT  
 AsnSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIleSer  
 CTTAAATTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAA  
 LeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyLysThrGluGln  
 GGCTTAAGTCCAAATCAAACCATTGCTACTGATAGTGGCGCGATGCCACATAAACTT  
 GlyLeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetProHisLysLeu  
 GAAAAAGCTACTTAAAGGCTATTCAAGAACAAATTGATCGCTAACGTCCACAGTAAC  
 GluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSerAsn  
 GACGACTACTTGAGGTATTGATTTGCAAGCGATGCAACCATTACTGATCGAAACGGC  
 AspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsnGly  
 AAGGTCTACTTGCTGACAAAGATGGTTGGTAACCTGCCAACCAACCTGTCCAAGAA  
 LysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProValGlnGlu  
 TTTTGCTAAGCGGACATGTGCGCTTAGACCATAAGAAAAACCAATACAAATCAA  
 PheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGlnAsnGln

**SUBSTITUTE SHEET**

GCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCCGTATGACGAT  
 AlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAspAspAsp  
 TTCAGACCAGGTCTCAAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACCATC  
 PheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThrIle  
 ACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTTAACAAAACCCATCCAGGCTAT  
 ThrSerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGlyTyr  
 ACGATTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTCGGTACGATT  
 ThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArgThrIle  
 TTACCAATGGATCAAGAGTTACTTACCATGTCAAAATCGGGAACAGCTTATGAGATC  
 LeuProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGluIle  
 AATAAAAAATCTGGTCTGAATGAAGAAATAACAAACACTGACCTGATCTCTGAGAAATAT  
 AsnLysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLysTyr  
 TACGTCTTAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCACTTGAAACTG  
 TyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeuLysLeu  
 TTCACCATCAAATACGTTGATGTCACACCAACGAATTGCTAAAAGCGAGCAGCTTTA  
 PheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeuLeu  
 ACAGCTAGCGAACGTAACCTAGACTTCAGAGATTACGATCCTCGTGATAAGGCTAAA  
 ThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAlaLys  
 CTACTCTACAACAATCTCGATGCTTGGTATTATGGACTATACTTAACGGAAAGTA  
 LeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLysVal  
 GAAGATAATCACGATGACACCAACCGTATCATAACCGTTATATGGCAAGCGACCCGAA  
 GluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArgProGlu  
 GGAGAGAATGCTAGCTATCATTAGCCTATGATAAAAGATCGTTACCGAAGAACGAA  
 GlyGluAsnAlaSerTyrHisLeuAlaTyrAspLysAspArgTyrThrGluGluArg  
 GAAGTTTACAGCTACCTGCGTTATACAGGGACACCTATACCTGATAACCCCTAACGACAAA  
 GluValTyrSerTyrLeuArgTyrThrGlyThrProIleProAspAsnProAsnAspLys  
 TAAGGATCC\*  
 End

\*\*\*\*\* END OF SEQ ID NO: 29 \*\*\*\*\*

**SUBSTITUTE SHEET**

SEQ. ID NO:33

SEQUENCE TYPE: nucleotide with corresponding amino acid  
SEQUENCE LENGTH: 2254 nucleotides  
FEATURES: Met-corestreptokinase-corestreptokinase  
fusion linked by thrombin-cleavable  
VELQGVVPRG

## SEQUENCE:

CATATGAGCCAATTAGTTGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATT  
MetSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIle  
AGTCTTAAATTTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAG  
SerLeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGlu  
CAAGGCTTAAGTCCAAAATCAAACCATTGCTACTGATAAGTGGCGCGATGCCACATAAA  
GlnGlyLeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetProHisLys  
CTTGAAAAAGCTGACTTACTAAAGGCTATTCAAGAACAAATTGATCGCTAACGTCCACAGT  
LeuGluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSer  
AACGACGACTACTTGAGGTATTGATTTGCAAGCGATGCAACCATTACTGATCGAAC  
AsnAspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsn  
GGCAAGGTCTACTTGCTGACAAAGATGGTCGTAACCTGCCGACCCAACCTGTCCAA  
GlyLysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProValGln  
GAATTTGCTAAGCGGACATGTGCGCGTTAGACCATAAAGAAAAACCAATACAAAAT  
GluPheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGlnAsn  
CAAGCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCCCTGATGAC  
GlnAlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAspAsp  
GATTTCAGACCAGGTCTCAAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACC  
AspPheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThr  
ATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTAAACAAAACCCATCCAGGC  
IleThrSerGlnGluLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGly  
TATACGATTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTCCTGAC  
TyrThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArgThr  
ATTTTACCAATGGATCAAGAGTTACTTACCATGTCAAAATCGGAACAAGCTTATGAG  
IleLeuProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGlu  
ATCAATAAAAATCTGGTCTGAATGAAGAAATAAACAAACACTGACCTGATCTCTGAGAAA  
IleAsnLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLys

SUBSTITUTE SHEET

TATTACGTCTTAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCACTGAAA  
 TyrTyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeuLys  
 CTGTTCACCATCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTC  
 LeuPheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeu  
 TTAACAGCTAGCGAACGTAACCTAGACTCAGAGATTACGATCCTCGTGATAAGGCT  
 LeuThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAla  
 AAACTACTCTACAACAATCTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAAA  
 LysLeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLys  
 GTAGAAGATAATCACGATGACACCAACCGTATCATAACCGTTATATGGGCAAGCGACCC  
 ValGluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArgPro  
 GAAGGAGAGAATGCTAGCTATCATTAGCCGTAGAGCTGCAGGGAGTAGTTCTCGTGGAA  
 GluGlyGluAsnAlaSerTyrHisLeuAlaValGluLeuGlnGlyValValProArgGly  
 AGCCAATTAGTTGTTAGCGTTGGTACTGTTGAGGGGACCAATCAAGACATTAGTCTT  
 SerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGlnAspIleSerLeu  
 AAATTTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGC  
 LysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLysThrGluGlnGly  
 TTAAGTCCAAAATCAAAACCATTGCTACTGATAGTGGCGCGATGCCACATAAAACTGAA  
 LeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetProHisLysLeuGlu  
 AAAGCTGACTTAAAGGCTATTCAAGAACATTGATCGCTAACGTCCACAGTAACGAC  
 LysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSerAsnAsp  
 GACTACTTGAGGTCAATTGATTGATTTGCAAGCGATGCAACCATTACTGATCGAAACGGCAAG  
 AspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAspArgAsnGlyLys  
 GTCTACTTGCTGACAAAGATGGTTGGTAACTGCTGCGACCCAACTGTCCAAGAATT  
 ValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnProValGlnGluPhe  
 TTGCTAACGGGACATGTGCGCGTTAGACCATAAAAGAAAAACCAATACAAAATCAAGCG  
 LeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIleGlnAsnGlnAla  
 AAATCTGTTGATGTGAAATATACTGTACAGTTACTCCCTTAAACCTGATGACGATTTC  
 LysSerValAspValGluTyrThrValGlnPheThrProLeuAsnProAspAspAspPhe  
 AGACCAGGTCTCAAAGATACTAACGCTATTGAAAACACTAGCTATCGGTGACACCACACA  
 ArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThrIleThr  
 TCTCAAGAATTACTAGCTCAAGCACAAAGCATTTAAACAAACCCATCCAGGCTATACG  
 SerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGlyTyrThr

## SUBSTITUTE SHEET

ATTTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTCGGTACGATTTA  
IleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePheArgThrIleLeu  
CCAATGGATCAAGAGTTACTTACCATGTCAAAAATCGGAACAAAGCTTATGAGATCAAT  
ProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGluIleAsn  
AAAAAAATCTGGTCTGAATGAAGAAATAAACAAACACTGACCTGATCTCTGAGAAATATTAC  
LysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLysTyrTyr  
GTCCTTAAAAAAGGGAAAAGCCGTATGATCCCTTGATCGCAGTCAC TGAAACTGTT  
ValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHisLeuLysLeuPhe  
ACCATCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTCTAAC  
ThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeuLeuThr  
GCTAGCGAACGTAACCTAGACTTCAGAGATTATACGATCCTCGTGATAAGGCTAAACTA  
AlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAspLysAlaLysLeu  
CTCTACAACAATCTGATGCTTTGGTATTATGGACTATACTGAAAGTAGAA  
LeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLysValGlu  
GATAATCACGATGACACCAACCGTATCATAACCGTTATATGGCAAGCGACCGAAGGA  
AspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLysArgProGluGly  
GAGAATGCTAGCTATCATTAGCCTAAGGATCC  
GluAsnAlaSerTyrHisLeuAlaEnd

\*\*\*\*\* END OF SEQ ID NO: 33 \*\*\*\*\*

## SUBSTITUTE SHEET

SEQ. ID NO:35

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1459 nucleotides  
 FEATURES: Hirudin-streptokinase fusion  
 linked by Factor Xa-cleavable IEGR  
 SEQUENCE:

GTTGTTTACACCGACTGTACTGAATCCGGACAAACCTGTGTTGTGAGGGTTCTAAC  
 ValValTyrThrAspCysThrGluSerGlyGlnAsnLeuCysLeuCysGluGlySerAsn  
 GTCTGTGGTCAGGGTAACAAATGCATCCTGGGTTCCGACGGTAAAAAGAACCAATGTGTC  
 ValCysGlyGlnGlyAsnLysCysIleLeuGlySerAspGlyGluLysAsnGlnCysVal  
 ACTGGTGAAAGGTACCCCAAAGCCGCAGTCCCACAACGATGGAGATTGCAAGAAATCCA  
 ThrGlyGluGlyThrProLysProGlnSerHisAsnAspGlyAspPheGluGluIlePro  
 GAAGAATATCTGCAGATCGAAGGTAGAATTGCTGGACCTGAGTGGCTGCTAGACCGTCCA  
 GluGluTyrLeuGlnIleGluGlyArgIleAlaGlyProGluTrpLeuLeuAspArgPro  
 TCTGTCAACAAACAGCCAATTAGTTGTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAA  
 SerValAsnAsnSerGlnLeuValValSerValAlaGlyThrValGluGlyThrAsnGln  
 GACATTAGTCTTAAATTTTGAAATTGACCTAACATCACGACCTGCTCATGGAGGAAAG  
 AspIleSerLeuLysPhePheGluIleAspLeuThrSerArgProAlaHisGlyGlyLys  
 ACAGAGCAAGGCTAACGACTAAAGTCCAAAATCAAACCATTGCTACTGATAGTGGCGCGATGCCA  
 ThrGluGlnGlyLeuSerProLysSerLysProPheAlaThrAspSerGlyAlaMetPro  
 CATAAACTTGAAAAAGCTGACTTACTAAAGGCTATTCAAGAACAAATTGATCGCTAACGTC  
 HisLysLeuGluLysAlaAspLeuLeuLysAlaIleGlnGluGlnLeuIleAlaAsnVal  
 CACAGTAACGACGACTTTGAGGTCAATTGATTTGCAAGCGATGCAACCATTACTGAT  
 HisSerAsnAspAspTyrPheGluValIleAspPheAlaSerAspAlaThrIleThrAsp  
 CGAAACGGCAAGGTCTACTTGCTGACAAAGATGGTTCGGTAACCTGCCGACCCAACCT  
 ArgAsnGlyLysValTyrPheAlaAspLysAspGlySerValThrLeuProThrGlnPro  
 GTCCAAGAATTTTGCTAACGGACATGTGCGCGTTAGACCATATAAAGAAAAACCAATA  
 ValGlnGluPheLeuLeuSerGlyHisValArgValArgProTyrLysGluLysProIle  
 CAAAATCAAGCGAAATCTGTTGATGTGGAATATACTGTACAGTTACTCCCTAAACCT  
 GlnAsnGlnAlaLysSerValAspValGluTyrThrValGlnPheThrProLeuAsnPro  
 GATGACGATTCAGACCAGGTCTCAAAGATACTAACGCTATTGAAAACACTAGCTATCGGT  
 AspAspAspPheArgProGlyLeuLysAspThrLysLeuLeuLysThrLeuAlaIleGly

SUBSTITUTE SHEET

GACACCATCACATCTCAAGAATTACTAGCTAACGCACAAAGCATTAAACAAAACCCAT  
 AspThrIleThrSerGlnGluLeuLeuAlaGlnAlaGlnSerIleLeuAsnLysThrHis  
  
 CCAGGCTATACGATTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATTTTC  
 ProGlyTyrThrIleTyrGluArgAspSerSerIleValThrHisAspAsnAspIlePhe  
  
 CGTACGATTTACCAATGGATCAAGAGTTACTTACCATGTCAAAAATCGGGAACAGCT  
 ArgThrIleLeuProMetAspGlnGluPheThrTyrHisValLysAsnArgGluGlnAla  
  
 TATGAGATCAATAAAAATCTGGTCTGAATGAAGAAATAAACACACTGACCTGATCTCT  
 TyrGluIleAsnLysLysSerGlyLeuAsnGluGluIleAsnAsnThrAspLeuIleSer  
  
 GAGAAATATTACGTCTTAAAAAAGGGGAAAGCCGTATGATCCCTTGATCGCAGTCAC  
 GluLysTyrTyrValLeuLysLysGlyGluLysProTyrAspProPheAspArgSerHis  
  
 TTGAAACTGTTCACCATCAAATACGTTGATGTCAACACCAACGAATTGCTAAAAGCGAG  
 LeuLysLeuPheThrIleLysTyrValAspValAsnThrAsnGluLeuLeuLysSerGlu  
  
 CAGCTCTAACAGCTAGCGAACGTAACCTAGACTTCAGAGATTACGATCCTCGTGAT  
 GlnLeuLeuThrAlaSerGluArgAsnLeuAspPheArgAspLeuTyrAspProArgAsp  
  
 AAGGCTAAACTACTCTACAACAATCTCGATGTTGGTATTATGGACTATACTTAAC  
 LysAlaLysLeuLeuTyrAsnAsnLeuAspAlaPheGlyIleMetAspTyrThrLeuThr  
  
 GGAAAAGTAGAAGATAATCACGATGACACCAACCGTATCATAACCGTTATATGGCAAG  
 GlyLysValGluAspAsnHisAspAspThrAsnArgIleIleThrValTyrMetGlyLys  
  
 CGACCCGAAGGAGAGAATGCTAGCTATCATTAGCCTATGATAAAGATCGTTATACCGAA  
 ArgProGluGlyGluAsnAlaSerTyrHisLeuAlaTyrAspLysAspArgTyrThrGlu  
  
 GAAGAACGAGAAGTTACAGCTACCTGCGTTATACAGGGACACCTATACCTGATAACCCT  
 GluGluArgGluValTyrSerTyrLeuArgTyrThrGlyThrProIleProAspAsnPro  
  
 AACGACAAATAAGGATCC\*  
 AsnAspLysEnd

\*\*\*\*\* END OF SEQ ID NO: 35 \*\*\*\*\*

**SUBSTITUTE SHEET**

SEQ. ID NO:38

SEQUENCE TYPE: nucleotide with corresponding amino acid  
 SEQUENCE LENGTH: 1468 nucleotides  
 FEATURES: Streptokinase-hirudin fusion  
 linked by Factor Xa-cleavable IEGR  
 SEQUENCE:

ATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAACAAACAGCCAATTAGTT  
 IleAlaGlyProGluTrpLeuLeuAspArgProSerValAsnAsnSerGlnLeuVal  
 GTTAGCGTTGCTGGTACTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATTTTGAA  
 ValSerValAlaGlyThrValGluGlyThrAsnGlnAspIleSerLeuLysPhePheGlu  
 ATTGACCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCAAAAA  
 IleAspLeuThrSerArgProAlaHisGlyGlyLysThrGluGlnGlyLeuSerProLys  
 TCAAAACCATTGCTACTGATACTGGCGCGATGCCACATAAACTGAAAAAGCTGACTTA  
 SerLysProPheAlaThrAspSerGlyAlaMetProHisLysLeuGluLysAlaAspLeu  
 CTAAAGGCTATTCAAGAACAAATTGATCGCTAACGTCCACAGTAACGACGACTACTTGAG  
 LeuLysAlaIleGlnGluGlnLeuIleAlaAsnValHisSerAsnAspAspTyrPheGlu  
 GTCATTGATTGCAAGCGATGCAACCATTACTGATCGAAAACGGCAAGGTCTACTTGCT  
 ValIleAspPheAlaSerAspAlaThrIleThrAspArgAsnGlyLysValTyrPheAla  
 GACAAAGATGGTCGGTAACCTTGCACCCAACTGTCCAAGAATTTTGCTAACGGGA  
 AspLysAspGlySerValThrLeuProThrGlnProValGlnGluPheLeuLeuSerGly  
 CATGTGCGCGTTAGACCATAAAAGAAAAACCAATAACAAATCAAGCGAAATCTGTTGAT  
 HisValArgValArgProTyrLysGluLysProIleGlnAsnGlnAlaLysSerValAsp  
 GTGGAATATACTGTACAGTTACTCCCTAACCCCTGATGACGATTTCAGACCAGGTCTC  
 ValGluTyrThrValGlnPheThrProLeuAsnProAspAspPheArgProGlyLeu  
 AAAGATACTAAGCTATTGAAAACACTAGCTATCGGTGACACCACATCACATCTCAAGAATTA  
 LysAspThrLysLeuLeuLysThrLeuAlaIleGlyAspThrIleThrSerGlnGluLeu  
 CTAGCTCAAGCACAAAGCATTTAAACAAAACCCATCCAGGCTATACGATTATGAAACGT  
 LeuAlaGlnAlaGlnSerIleLeuAsnLysThrHisProGlyTyrThrIleTyrGluArg  
 GACTCCTCAATCGTCACTCATGACAATGACATTTCCGTACGATTTCACCAATGGATCAA  
 AspSerSerIleValThrHisAspAsnAspIlePheArgThrIleLeuProMetAspGln  
 GAGTTTACTTACCATGTCAAAATCGGGAACAAGCTTATGAGATCAATAAAAATCTGGT  
 GluPheThrTyrHisValLysAsnArgGluGlnAlaTyrGluIleAsnLysSerGly

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CTGAATGAAGAAATAAACAAACACTGACCTGATCTCTGAGAAATATTACGTCTTAAAAAA  
LeuAsnGluGluIleAsnAsnThrAspLeuIleSerGluLysTyrTyrValLeuLysLys  
GGGGAAAAGCCGTATGATCCCTTGATCGCAGTCACTTGAAACTGTTCACCATCAAATAC  
GlyGluLysProTyrAspProPheAspArgSerHisLeuLysLeuPheThrIleLysTyr  
GTTGATGTCAACACCAACGAATTGCTAAAAGCGAGCAGCTCTAACAGCTAGCGAACGT  
ValAspValAsnThrAsnGluLeuLeuLysSerGluGlnLeuLeuThrAlaSerGluArg  
AACTTAGACTTCAGAGATTATACGATCCTCGTGATAAGGCTAAACTACTCTACAACAAT  
AsnLeuAspPheArgAspLeuTyrAspProArgAspLysAlaLysLeuLeuTyrAsnAsn  
CTCGATGCTTTGGTATTATGGACTATACCTTAACGGAAAGTAGAAGATAATCAGAT  
LeuAspAlaPheGlyIleMetAspTyrThrLeuThrGlyLysValGluAspAsnHisAsp  
GACACCAACC GTATCATAACCGTTATATGGGCAAGCGACCCGAACGGAGAGAATGCTAGC  
AspThrAsnArgIleIleThrValTyrMetGlyLysArgProGluGlyGluAsnAlaSer  
TATCATTAGCCTATGATAAAAGATCGTTACCGAAGAACGAGAAGTTACAGCTAC  
TyrHisLeuAlaTyrAspLysAspArgTyrThrGluGluGluArgGluValTyrSerTyr  
CTGCCTTATACAGGGACACCTATACCTGATAACCTAACGACAAAATCGAAGGTAGAGTT  
LeuArgTyrThrGlyThrProIleProAspAsnProAsnAspLysIleGluGlyArgVal  
GTTTACACCGACTGTACTGAATCCGGACAAACCTGTGTTGTGAGGGTTCTAACGTC  
ValTyrThrAspCysThrGluSerGlyGlnAsnLeuCysLeuCysGluGlySerAsnVal  
TGTGGTCAGGGTAACAAATGCATCCTGGGTTCCGACGGTGAAAAGAACCAATGTGTCAC  
CysGlyGlnGlyAsnLysCysIleLeuGlySerAspGlyGluLysAsnGlnCysValThr  
GGTGAAGGTACCCAAAGCCGCAGTCCCACAACGATGGAGATTCGAAGAAATCCCAGAA  
GlyGluGlyThrProLysProGlnSerHisAsnAspGlyAspPheGluGluIleProGlu  
GAATATCTGCAGTAATAGGGATCCGAATTC\*  
GluTyrLeuGlnEndEnd

\*\*\*\*\* END OF SEQ ID NO: 38 \*\*\*\*\*

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CLAIMS

1. A fusion protein comprising a first sequence and a second sequence, the fusion protein being cleavable between the first and second sequences by an enzyme involved in blood clotting, wherein after the fusion protein is so cleaved the first and second sequences, or either of them, has greater fibrinolytic and/or anti-thrombotic activity than the uncleaved fusion protein.
2. A fusion protein as claimed in claim 1, which is a cleavable dimer of two fibrinolytic and/or anti-thrombotic proteins.
3. A fusion protein as claimed in claim 1 or 2, wherein the first sequence corresponds to a hirudin or to a protein having the activity of hirudin.
4. A fusion protein as claimed in claim 1 or 2, wherein the first sequence corresponds to streptokinase or to a protein having the activity of streptokinase.
5. A fusion protein as claimed in any one of claims 1 to 4, wherein the second sequence corresponds to a hirudin or to a protein having the activity of hirudin.
6. A fusion protein as claimed in any one of claims 1 to 4, wherein the second sequence corresponds to streptokinase or to a protein having the activity of streptokinase.

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7. A fusion protein as claimed in any one of claims 1 to 6, wherein the enzyme involved in blood clotting is kallikrein, Factor XIIa, XIa, IXa, VIIa, Xa, thrombin (Factor IIa) or activated protein C.

8. A fusion protein as claimed in any one of claims 1 to 6, wherein the enzyme involved in blood clotting is Factor Xa or thrombin.

9. A fusion protein as claimed in any one of claims 1 to 6, wherein the enzyme involved in blood clotting is Factor Xa.

10. A fusion protein as claimed in claim 9, comprising the cleavage site sequence P4-P3-Gly-Arg, wherein P4 represents a hydrophobic residue and P3 represents an acidic residue.

11. A fusion protein as claimed in claim 10, wherein the hydrophobic residue is isoleucine.

12. A fusion protein as claimed in any one of claims 1 to 6, wherein the enzyme involved in blood clotting is thrombin.

13. A fusion protein as claimed in claim 12, comprising the cleavage site sequence P4-P3-Pro-Arg-P1'-P2', wherein each of P4 and P3 independently represents a hydrophobic residue and each of P1' and P2' independently represents a non-acidic residue.

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14. A fusion protein as claimed in claim 12, comprising the cleavage site sequence P2-Arg-P1', wherein one of the residues P2 and P1' represents glycine, and the other is any amino acid residue.
15. A fusion protein as claimed in claim 12, comprising the cleavage site sequence Gly-Pro-Arg.
16. A process for the preparation of a fusion protein as claimed in any one of claims 1 to 15, the process comprising coupling successive amino acid residues together and/or ligating oligo- and/or poly-peptides.
17. Synthetic or recombinant nucleic acid coding for a fusion protein as claimed in any one of claims 1 to 15.
18. Nucleic acid as claimed in claim 17, which is a vector.
19. A process for the preparation of nucleic acid as claimed in claim 17, the process comprising coupling successive nucleotides together and/or ligating oligo- and/or poly-nucleotides.
20. A cell or cell line transformed or transfected with a vector as claimed in claim 18.
21. A cell as claimed in claim 20, which is a yeast cell.
22. A yeast cell as claimed in claim 21 which is Pichia pastoris or Saccharomyces cerevisiae.

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23. A cell as claimed in claim 20, which is a bacterial cell.

24. A bacterial cell as claimed in claim 23, which is Escherichia coli.

25. A pharmaceutical composition comprising one or more compounds as claimed in any one of claims 1 to 15 and a pharmaceutically or veterinarily acceptable carrier.

26. A method for the treatment or prophylaxis of thrombotic disease, the method comprising the administration of an effective, non-toxic amount of a fusion protein as claimed in any one of claims 1 to 15.

27. A proteinaceous compound as claimed in any one of claims 1 to 15 for use in human or veterinary medicine.

28. The use of a fusion protein as claimed in any one of claims 1 to 15 in the preparation of a thrombolytic and/or antithrombotic agent.

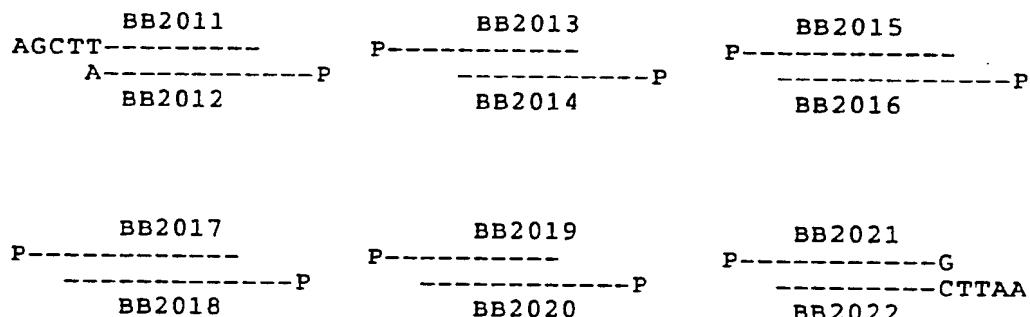
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## FIG. 1.

## SUMMARY OF ASSEMBLY PROCEDURE

The kinased oligomers were annealed in pairs. The oligomers BB2011 and BB2020 were not kinased to prevent multimerization.

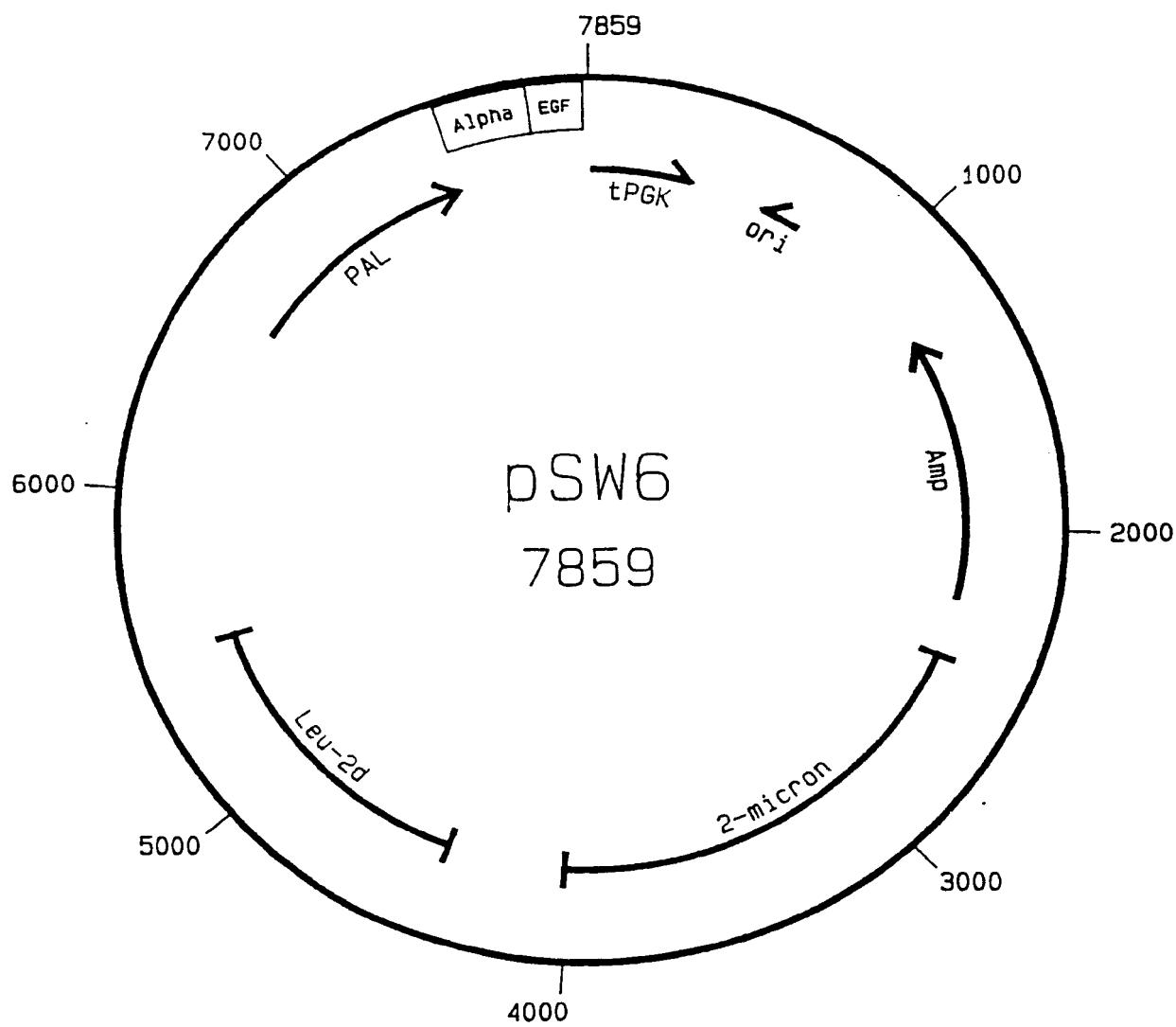


p=5' phosphate

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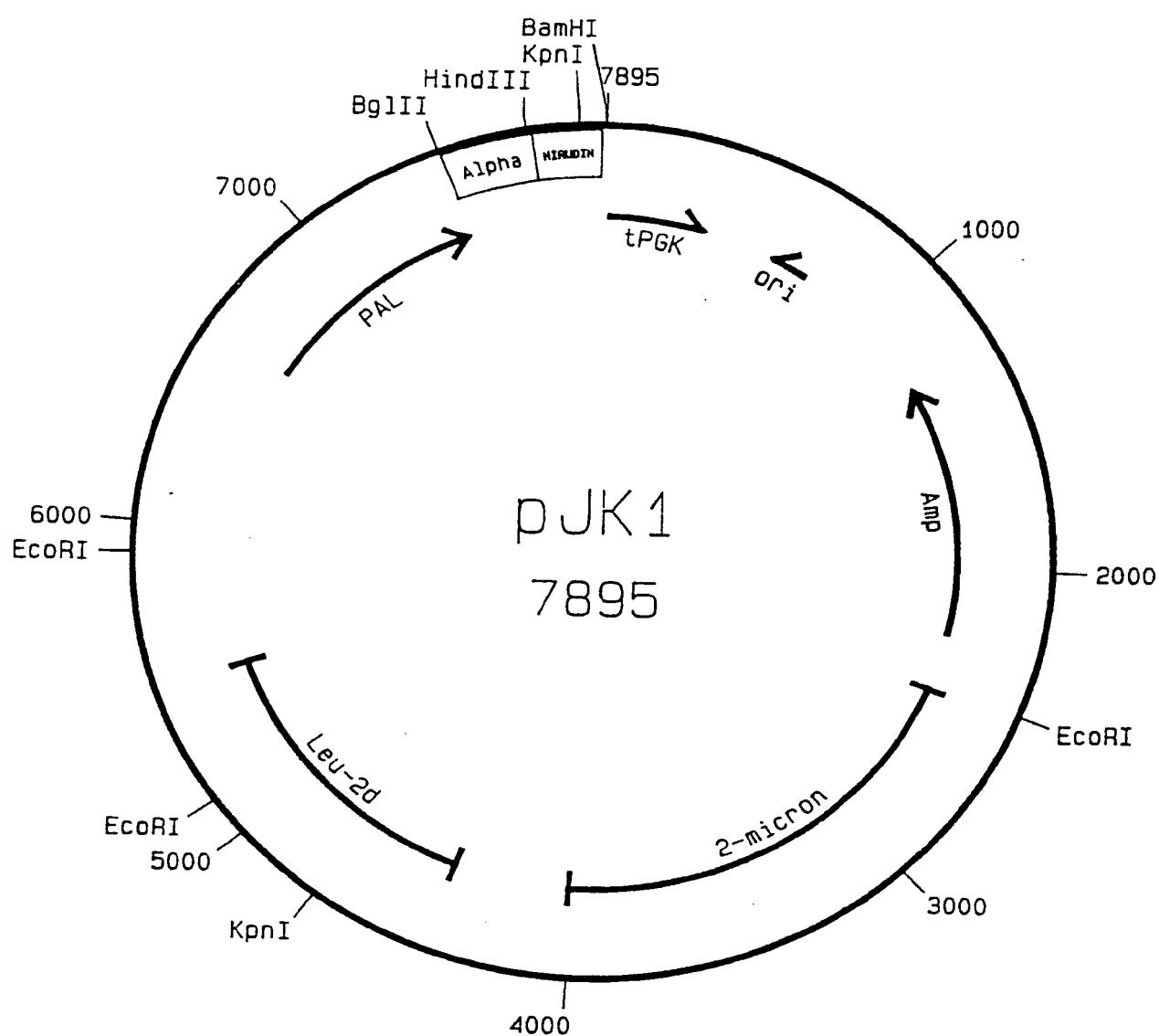
FIG. 2.



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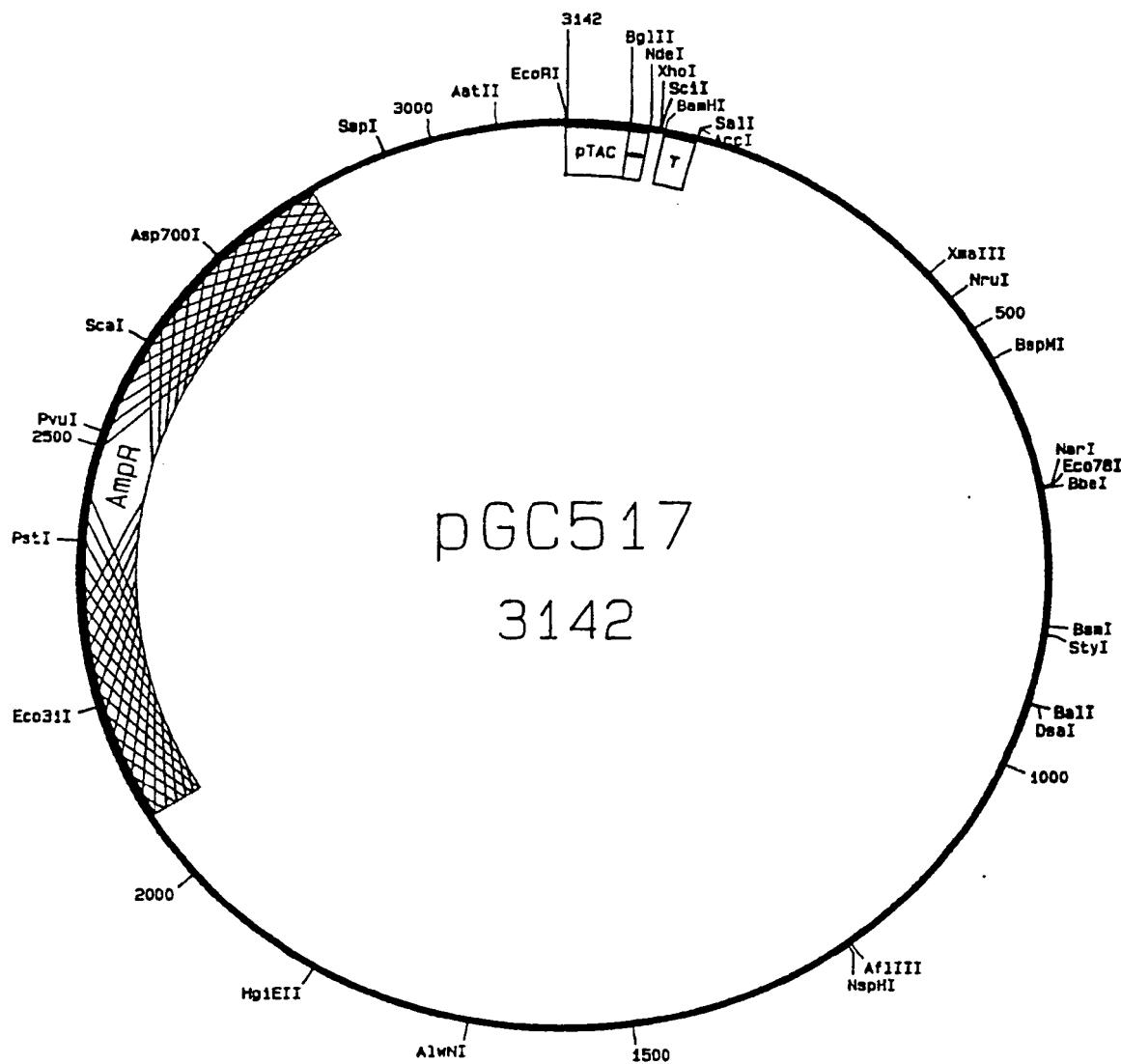
FIG. 3.



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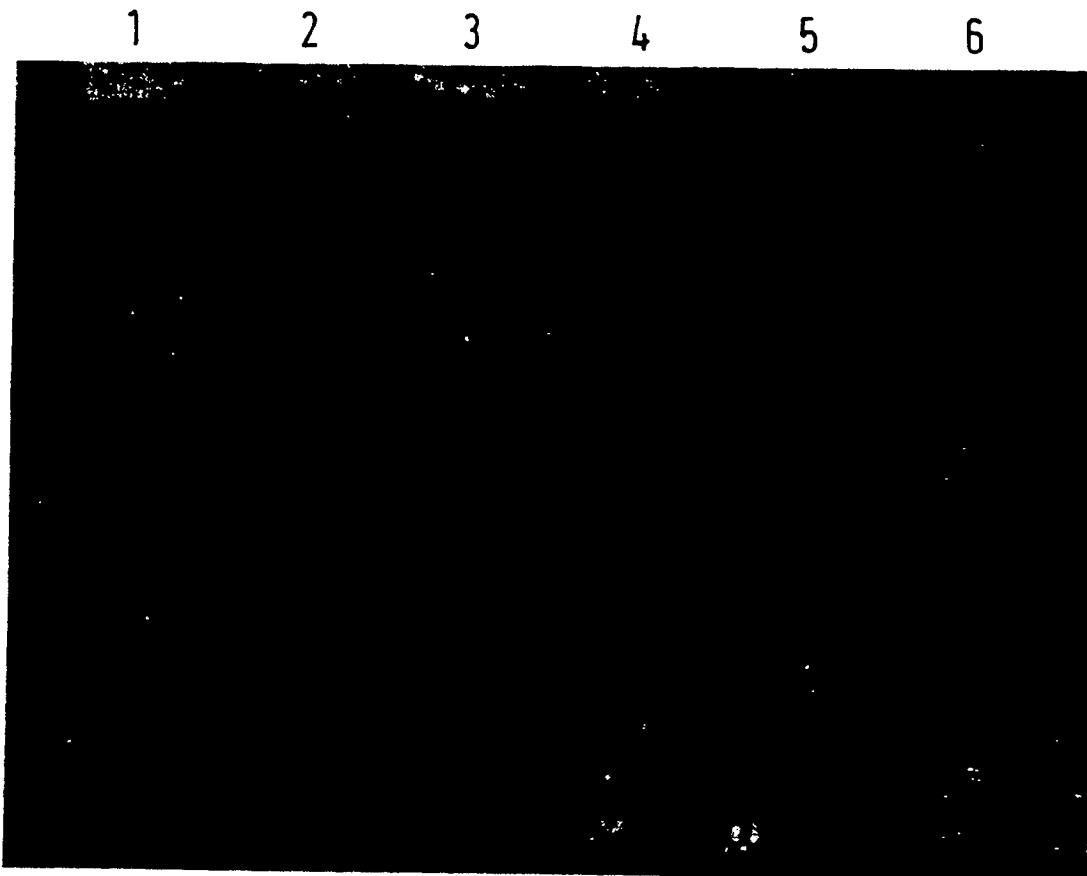
FIG. 4.



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## FIG. 5.



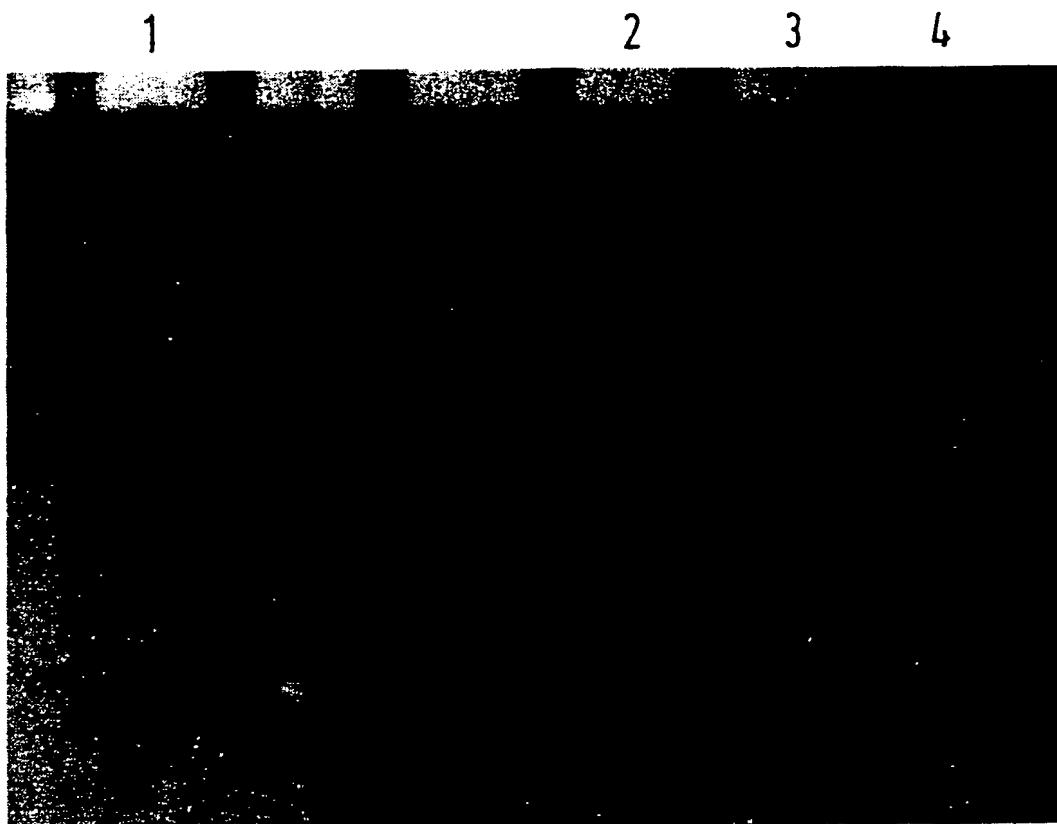
Zymograph of E. coli strains expressing streptokinase activity.

Lane 1. E. coli JM103 pKJ2 uninduced. Lane 2. E. coli JM103 pKJ2 IPTG induced. Lane 3. E. coli HW1110 pLGC1 uninduced. Lane 4. E. coli HW1110 pLGC1 IPTG induced. Lane 5. E. coli HW1110 pLGC2 uninduced. Lane 6. E. coli HW1110 pLGC2 IPTG induced.

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FIG. 6.



Zymograph of in vitro cleavage of the thrombin cleavable Streptokinase-streptokinase molecule by thrombin.

Lane 1, Streptokinase. Lane 2, 15-40% cut containing high molecular weight streptokinase activity, no thrombin. Lane 3, as 2 but 0.5 U/ml thrombin. Lane 4, as 2 but 5 U/ml thrombin.

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 90/01911

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC: 15/58, 15/62, 9/70, C 07 K 7/10, C 12 N 5/10, C 12 N 15/15,  
 1/19, 1/21, A 61 K 37/64, 37/54

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
IPC <sup>5</sup>	C 12 N, C 07 K, A 61 K

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP, A, 0296413 (HOECHST) 28 December 1988 see example 6	1,7,8,12, 16-20,25, 27,28
Y	---	15
Y	EP, A, 0323149 (ELI LILLY) 5 July 1989 see page 13, lines 19-30; page 16, lines 5-13	15
X	EP, A, 0292009 (ZYMOGENETICS) 23 November 1988 see page 3, lines 1-31; page 3, lines 57-58; page 6, line 48 - page 7, line 8; page 8, line 45 - page 9, line 15; page 22, section D; page 7, line 54 - page 8, line 2, examples 8,10,11; page 26, lines 38-45	1,7,8,12, 16-25,27,28
Y	---	2 ./.

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

10th March 1991

Date of Mailing of this International Search Report

08.05.91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP, A, 0227938 (HOECHST) 8 July 1987 see page 3, lines 25-32; examples 1,2,4	3,9-12,25, 27,28
A	Mol Gen Genet, volume 212, 1988. MGG Springer-Verlag, C. Klessen et al.: "Tripartite streptokinase gene fusion vectors for gram-positive and gram-negative prokaryotes", pages 295-300 see the whole document	4
A	EP, A, 0330700 (SAGAMI) 6 September 1989 see page 3, line 52 - page 4, line 45; page 5, line 20 - page 6, line 57	1,12

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET****V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1**

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers .26..... because they relate to subject matter not required to be searched by this Authority, namely:

see PCT Rule 39.1(iv)

2.  Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2**

This International Searching Authority found multiple inventions in this international application as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.  As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

The additional search fees were accompanied by applicant's protest.  
 No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9001911  
SA 42783

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/04/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0296413	28-12-88	JP-A-	1085096	30-03-89
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EP-A- 0330700	06-09-89	WO-A-	8901513	23-02-89

